DEVELOPMENT OF INFECTIOUS PAPAYA MOSAIC VIRUS USING A cDNA CLONE FUSED TO THE 35S CaMV PROMOTER

By

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In memory of my grandmother, Teofila Betancourt

To my parents, for showing me the way.
To my sister, for being the best example to follow.
To my husband and children, for reminding me what is really important in life.

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KEY TO ABBREVIATIONS

ATCC	American Type Culture Collection
bp	base pair
CaMV35S	cauliflower mosaic virus 35S promoter
CP	coat protein
C-terminus	carboxy-terminus
cDNA	complementary DNA
CMV	cucumber mosaic virus
ELISA	enzyme-linked immunosorbent assay
HC/Pro	helper component/protease
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
LB	Luria broth
β-ME	β-mercaptoethanol
MW	molecular weight
3'-NCR	3' non-coding region
N-terminus	amino-terminus
nm	nanometer
nt	nucleotide
oligo dT	oligonucleotide deoxythymidine
PCR	polymerase chain reaction
PDR	pathogen derived resistance
PMV	papaya mosaic virus
psi	pounds per square inch
PRSV-W	papaya ringspot virus type W
PRSV-P	papaya ringspot virus type P
PVY	potato virus Y
RT-PCR	reverse transcription-polymerase chain reaction
TMV	tobacco mosaic virus
X-Gal	5-bromo-4-chloro-3-infolyl-β-D-galactopyranoside
KPa	kilopascals

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The objective of this research project has been to develop an infectious clone of papaya mosaic virus (PMV; genus Potexvirus) so that it can be engineered as a vector for gene expression in papaya, primarily to express papaya ringspot virus (PRSV; genus Potyvirus) genes for pathogen-derived resistance. The vector will circumvent the lengthy process of incorporating foreign genes into the papaya genome. With the vector it should be possible to mechanically inoculate papaya plants and rapidly test for gene expression. PMV was chosen as a vector, because it produces mild symptoms in papaya and is not known to be transmitted by insects, reducing the ecological risk of spreading transgenes into the environment. In order to achieve this goal, the first step was to develop infectious clones of PMV. Full-length clones of PMV were generated from cDNA of PMV purified from systemically infected leaves of *Carica papaya* L. After RNA extraction and

purification, Superscript II, RNase H-reverse transcriptase was used for first strand synthesis. Second strand synthesis was accomplished using primers specific for the 5' and 3' ends using the Long Template PCR System. The cDNAs obtained were cloned into the pGEM-TEasy vector. The 35 S promoter from the plasmid pCambia 1302 was attached directly to the 5' end of the PMV clone using a series of overlapping PCR steps. The resulting construct was then ligated into pGEM-T Easy and amplified using Sure2 *E. coli* ultracompetent cells. Plasmids containing the full-length construct were selected, purified and used either whole or linearized to mechanically inoculate papaya seedlings. One infectious clone produced typical PMV systemic symptoms in C. *papaya* and local lesions in *Gomphrena globosa.* These results were confirmed by tissue blots, ELISA and electron microscopy of symptomatic tissue.

CHAPTER 1
INTRODUCTION

Papaya ringspot virus (PRSV-P) causes a major disease in papaya (*Carica papaya*), that greatly limits the production of high quality papaya. It occurs in most regions of the tropical world where papaya is grown with the exception of Africa. The virus is a member of the family Potyviridae, genus potyvirus. Aphids spread the virus from plant to plant within an orchard. The virus is not transmitted through seed. It is transmitted nonpersistently by aphids and is also mechanically transmissible by sap inoculation. PRSV-P infects certain members of the Chenopodiaceae and Cucurbitaceae. Two strains of the virus have been designated: PRSV-P, which infects papaya and cucurbits (cucumber, pumpkin, squash and watermelon), and PRSV-W, which infects cucurbits but not papaya (Purcifull et al., 1984). Symptoms, which may vary depending on the papaya variety, include dark green rings on the fruit, yellow mosaic on leaves and "oily" streaks on petioles. Some leaves may develop shoestring symptoms and otherwise be distorted. Infected papaya produces less fruit, and the fruit has a different texture and lower sugar content. A few tolerant varieties are available but the degree of tolerance is not adequate. In many cases, tolerant varieties have inferior commercial qualities. Recently, virus-resistant transgenic papaya has been produced in Hawaii (Gonsalves, 1998) and Florida (Davis and Ying, 1999).

Papaya General Characteristics

Papaya a member of the family Caricaceae is a tropical fruit with its center of origin in Tropical America (Manshardt, 1992). It is widely distributed throughout the tropics, probably aided by an abundance of seed of relatively long viability (up to three years under cool, dry conditions). It is now naturalized in many tropical regions, particularly in areas with fertile soils and abundant rainfall. The tree produces three different kinds of flowers: hermaphroditic, pistillate, and staminate. The wild type papaya is generally dioecious, but domestication has favored gynodioecious forms. This is due to the production of more predictable characteristics in the progenies (Storey, 1976). Seeds take about two weeks to germinate. The plants flower after 6-7 months, and the fruit is harvested between 9-11 months after planting. Papaya is consumed as a fruit for dessert (fresh or preserved in sugar) or as a green vegetable. Numerous commercial processed products have been developed and are currently quite popular. Some of the diseases affecting papaya in Florida and the Caribbean include PRSV, papaya bunchy top and several types of root and fruit rots (Hepperly, 1994). These and other diseases can be a limiting factor in the production of high quality fruits.

Economic Importance of Papaya Ringspot Virus

Worldwide papaya production was over 5 million metric tons for 1998; the USA produced 18,140 metric tons and 1,905 metric tons were produced in Puerto Rico (FAO statistics). Imports of the fruit in the USA during that same year (48,201 metric tons) were worth $28,519,000 and 6,788 metric tons worth $16,730,000 were exported. The biggest producer for the U. S. market is Hawaii. In 1995-96, Florida produced 2,835 tons

(Degner et al., 1997) with an annual estimated worth of $ 2-4 million.

General Characteristics of Potyviruses

The family *Potyviridae* is one of the largest of plant virus groups recognized by the International Committee for the Taxonomy of viruses. There are three recognized genera: *Potyvirus*, *Rymovirus* and *Bymovirus* (Murphy et al., 1995). Most potyviruses (*Potyvirus*) are transmitted by aphids in a non-persistent manner, while Rymoviruses are transmitted by mites and Bymoviruses are transmitted by fungi. Potyviruses induce characteristic cylindrical inclusion bodies in the cytoplasm of infected cells (Edwardson, 1974). The cylindrical inclusions are formed by a virus-encoded protein (Dougherty and Hiebert, 1980).

Genome Organization and Viral Replication

Potyvirus particles consist of one positive sense, single stranded genome of 8.5-10.5 kb encapsidated by a single type of coat protein (Hollings and Brunt, 1981). At the 5' end the RNA has a protein (VPg) covalently attached (Riechmann et al., 1989; Murphy et al., 1990). At the 3' end it has polyadenylated tract (Hari, 1979; Riechmann et al., 1992). The genomes encode a single protein that is co- or post-translationally processed to yield between seven to eleven different virus-encoded gene products. The products include three proteinases that catalyze the proteolytic cleavages that will result in the different virus encoded proteins. As a single stranded positive sense RNA, the genome acts as a messenger RNA and is translated immediately after infection of the host cell.

Papaya Ringspot Virus

The complete nucleotide sequence of the RNA genome of papaya ringspot virus has been determined for a Hawaiian (Yeh et al., 1992) isolate and for a Taiwanese isolate (Wang and Yeh, 1997). The genomic RNA is 10,326 nucleotides long (not including the poly A tract). It contains one large open reading frame (ORF) that encodes a protein of 3,344 amino acids. It has a highly conserved sequence at the 5' end of the RNA, which shows similarity to other potyviruses, and this suggests that the region may have a role in replication. PRSV has a genetic organization that is similar to that of other potyviruses, but the first protein processed from the N-terminus of the polyprotein (NT protein) has a MW of 63 K, which is 18 to 34 K larger than the ones of other potyviruses. The most conserved protein of potyviruses is the nuclear inclusion (NIb) protein, the polymerase for the replication of the viral RNA.

Isolates of PRSV from the Caribbean, Hawaii and North America appear to be closely related to each other as compared to isolates from Asia. Geographic origin rather than host specificity accounts for the differences so far observed in the phylogenetic analysis of the coat protein of PRSV (Bateson et al., 1994; Wang et al. 1994; Davis and Ying, 1999).

Management of PRSV

Gonsalves (1998) has recently summarized the current strategies for management of PRSV. In places where the disease is endemic, control efforts have included rouging,

cultural practices, breeding for resistance to PRSV and cross-protection (Gonsalves, 1994). Unfortunately, these methods provide only limited control of the disease.

In 1986 Conover et al. selected a tolerant dioecious variety, 'Cariflora', from which further tolerant varieties have been developed. The tolerance appears to be polygenic and is inherited quantitatively (Conover and Litz, 1978). Other tolerant papaya varieties have been developed in breeding programs (Crane et al., 1995; Manshardt and Zee, 1994; and Wang, 1982). The tolerant varieties, when infected with exhibit milder symptoms and produce fewer fruit but the fruit are more marketable. Attempts to obtain interspecific hybrids of *Carica papaya* with other members of the Caricaceae family that are immune to PRSV had limited success (Drew et al., 1998; Magdalita et al., 1998; Manshardt and Drew, 1998; Manshardt, 1992; Manshardt and Wenslaf, 1989a, 1989b).

Cross-Protection

The use of a mild strain to protect plants against damage caused by infection with a severe strain of the same virus has been used for the control of PRV in different parts of the world (McMillan and Gonsalves, 1988; Yeh et al., 1988; Yeh, 1990).

Cross-protection has been tested in Hawaii using the mild strain HA 5-1 in large field trials. Cross protection has allowed growers to remain productive even with high virus pressure (Mau et al., 1989). However, some cultivars, such as 'Sunrise' were found to be too sensitive to the mild strain to be protected commercially. In addition, there were yield losses of 10-15% and increased fertilizer and weed management was necessary due to decreased vigor of the cross-protected plants.

Tennant et al. (1994, 1997) tested papaya inoculated with the mild strain for cross protection against PRSV isolates from Florida, the Caribbean, Australia, China,

Mexico, South America, and Thailand. The protection ranged from attenuation of symptoms for many isolates to no detectable protection. Their conclusion was that the Hawaiian mild strain did not confer proper levels of protection against non-Hawaiian PRSV strains. In general, cross protection has been more effective in conjunction with cultural practices, such as rouging of PRV infected plants before the flowering stage and in papaya orchards isolated from severely infected trees.

Pathogen-Derived Resistance

Pathogen-derived resistance is based on the concept proposed by Sanford and Johnston (1985). The theory of pathogen–derived resistance (PDR) predicts that a normal host pathogen relationship can be disrupted if the host organism expresses pathogen-derived genes. It has been proposed that hosts expressing pathogen genes in excess amounts, at inappropriate developmental stages or in a defective form, may disrupt the replication cycle of the pathogen which results in an attenuated infection.

Transgenic plants expressing the coat protein of a plant virus can be resistant to infection by the virus. This type of resistance is known as coat protein mediated resistance. It has been demonstrated for several virus groups such as potexvirus, potyvirus, cucumovirus, tobravirus, tobamovirus, luteovirus and alfalfa mosaic virus groups (Beachy et al., 1990; Beachy, 1993, Beachy, 1997; Kawachuck et al., 1990, 1991; Lindbo and Dougherty, 1992a). Plants expressing a transgene from an RNA virus can be immune to the virus. This resistance is present with both translatable and untranslatable constructs indicating that the translation product of the transgene transcript is not necessarily involved in conferring resistance (Lindbo and Dougherty, 1992a, 1992b; van der Vlugt et al., 1992; Pang et al., 1993).

There are presently numerous examples where expression of viral sequences in a transgenic plant provided excellent resistance to subsequent viral infection (Lomonossof, 1995). Dougherty et al. (1994) have generated transgenic tobacco plants expressing various forms of the tobacco etch potyvirus (TEV) coat protein (CP) and found that plants expressing defective CP genes (untranslatable sense) had the greatest resistance. They proposed that natural cell defenses against deleterious overexpression of mRNA are involved. The untranslatable TEV CP mRNA are more readily dissociated from ribosomes than the translatable mRNAs and so become more accessible to the cellular degradation system.

Lindbo et al. (1993) indicated that transgenic tobacco plants expressing a full-length or truncated form of the TEV CP gene can recover from TEV infection. Further studies provided additional evidence that transgenic plants displaying RNA- mediated resistance phenotypes transcribe the transgene(s) at a high rate but accumulate the transgene transcript at low levels (English and Baulcombe, 1997). Results indicate that transcript level is inversely correlated with resistance and that untranslatable constructs give rise more frequently to highs of level resistance (Dougherty et al., 1994; Smith et al., 1994; Mueller et al., 1995; Swaney et al., 1995; Baulcombe, 1996; Goodwin et al., 1996).

Transgenic Resistance to Papaya Ringspot Virus

Papaya plants have been successfully genetically engineered for resistance to PRV in Hawaii (Gonsalves, 1998; Fitch et al., 1992). Transgenic papaya with resistance to PRV was first developed by Fitch et al. (1990) through microprojectile bombardment of embryogenic callus from tissue culture. They used the CP of strain HA 5-1 (Hawaiian

mild strain) in the translatable form. Transgenic papaya plants proved to be resistant after mechanical inoculation with PRV (Fitch et al., 1992; Slightom and Gonsalves, 1994). It was determined that the transgene in the modified plants was hemizygous, that the hemizygous gene conferred a narrow range of resistance to other PRV strains and that the resistance was most effective against closely related strains of PRV (Tennant et al., 1994; Yeh and Gonsalves, 1994). In the case of papaya, CP mediated resistance is broader when the transgene is homozygous (Tennant et al., 1994; Yeh et al., 1997). Wide range resistance may also be obtained if the untranslatable forms of the transgene are used. Papaya coat protein genes that have been genetically modified might confer broader ranges of resistance to strains from different geographic regions (Manshardt and Drew, 1998; Souza et al., 1998) thus, the effectiveness of the resistance is determined by factors such as origin, copy number, and gene dosage of the coat protein gene introduced into papaya. The use of transgenic papaya plants has had a positive impact in the Hawaiian papaya industry. These Hawaiian varieties cannot be commercially grown outside Hawaii due to licensing restrictions. The technology is not yet available to growers in the Caribbean.

Using reverse transcription polymerase chain reaction (RT-PCR) and sequencing techniques, Davis and Ying (1999) have examined the genetic diversity of PRV strains in South Florida and Puerto Rico. When the sequences were compared with those in available databases of 23 other strains from other parts of the world, it was found that the Florida strains are more similar to one another than to strains from other locations. The Puerto Rico and Florida strains were more closely related to one another than to strains from Mexico, Hawaii, Australia and more distantly related strains from Asia.

Ying et al. (1999a) developed an efficient method for *Agrobacterium*–mediated transformation and further regeneration of papaya (*Carica papaya* L. line F 65). Ying et al. (1999b) recently developed PRSV resistant papaya lines by transforming embryonic callus with constructs based on the coat protein (CP) gene of a Florida isolate. Four genetic constructs were used to transform papaya embryos. The constructs included sense, antisense, and untranslatable forms (frameshift and stop-codon mutations). They obtained 48 lines that appear to be highly resistant or immune to PRSV. Their research aims to improve commercial production of papaya in Florida and the Caribbean by developing papaya-breeding lines with transgenic PRV resistance. These geographic areas have particular fruit market needs and preferred characteristics.

Gene Silencing

Sometimes, virus-derived transgenes are expressed in a deficient manner and still provide virus resistance in plants. Transgene constructs designed to supplement the expression of endogenous genes have had an opposite effect such as co-suppressing themselves and the endogenous genes. These two phenomena apparently result from the same post-transcriptional gene silencing mechanism, which operates by targeted RNA degradation. The term 'gene silencing' comes from these observations of unpredictable silencing and variable expression and the phenomenon has been designated as homology-dependent gene silencing. The silencing process has implications for mechanisms of gene expression in plants and other eukaryotes.

In some cases, the mechanism of pathogen derived resistance involves transgenically expressed proteins. There are other examples in which the mechanism is

based on RNA. It appears that this mechanism is related to post-transcriptional gene silencing in transgenic plants.

Gene silencing in transgenic plants is expressed as a decrease in the accumulation of specific mRNAs and occurs most often when multiple copies of the transgene are present (Matzke et al., 1994.). Recent studies have linked post-transcriptional gene silencing (PTGS) with virus resistance in transgenic plants expressing virus-derived sequences. This association is based on observations that resistance has been achieved with untranslatable as well as translatable transgenes in plants that accumulate low levels of the transgene mRNA and protein product (Lindbo et al., 1993; Mueller et al., 1995). Non transgenic plants sometimes recover from viral infection by a PTGS-like mechanism. Primary infection in these plants is normal with the virus spreading systemically, but new leaves symptoms do not develop and viral RNA fails to accumulate because of RNA degradation (English et al., 1996; Ratcliffe et al., 1997). Transcriptional gene silencing (TGS) and PTGS are classified as two types of silencing under homology dependent gene silencing. Homology-dependent gene silencing represents a type of epigenetic inactivation that can occur when one or more copies of a particular sequence are present in the genome. Since the silencing results due to the presence of homologous or complementary nucleic acid sequences, it has been named homology-dependent–gene silencing (Kooter et al., 1999).

Baulcombe (1999) has suggested that gene silencing is likely to involve antisense RNA produced by the action of the host-encoded RNA-dependent RNA polymerase. The natural role of this mechanism is as a genetic immune system conferring protection against viruses.

Virus Vectors for the Expression of Foreign Genes

For the last 20 years, viruses have been studied as possible vectors for the expression of foreign proteins in plants. Much of this research has been motivated by the potential economic benefits of inexpensive, high level expression of valuable proteins in systems capable of producing high yields (e.g. the use of field crops as hosts for genetically engineered viruses that express foreign genes).

Early efforts to develop virus-based vectors for plant transformation focused on DNA, rather than RNA viruses. Studies from the early 1990s onwards have provided numerous examples demonstrating that plant RNA viruses are well suited to serve as transient vectors for foreign genes (Chapman et al., 1992; Dolja et al., 1992; Joshi et al., 1990; Takamatsu et al., 1987; Scholthof et al., 1993, 1996).

To develop more efficient systems for the high-level synthesis of specific foreign proteins in plants, attention has recently focused on high copy number RNA viruses as vectors for gene expression. Plant viruses offer numerous advantages as vehicles for transient expression of foreign genes, including their characteristically rapid multiplication to high levels with high-level expression. Maximum levels of foreign gene expression from a viral genome can occur within one or two weeks after inoculation. Another practical advantage is that many plant viruses can be transmitted mechanically, which can be used for commercial scale inoculation over large areas of crop plants.

In some viruses, foreign genes can be inserted that are under the transcriptional control of a viral subgenomic promoter sequence. Duplicating native viral subgenomic RNA promoters for foreign gene expression can lead to high frequencies of homologous recombination between duplicated sequences resulting in rapid deletion of the inserted

foreign gene (Dawson et al., 1989). One solution to this problem is to use a subgenomic RNA promoter from a closely related species, to avoid homologous recombination and thereby produce a more genetically stable vector for foreign gene expression (Donson et al., 1991). Examples of such vectors based on rod shaped viruses have been described for TMV,tobacco etch virus (TEV) and PVX.

Duplication of the homologous coat protein subgenomic promoter has proved to be an efficient way to express foreign genes from PVX without rapid recombination deletion. The gene insertion strategy has been used successfully with PVX to study virus infection (Baulcombe et al., 1995), for structure-function analyses of a *myb*-like transcription factor from potato (Sablowski et al., 1995), the avirulence gene *avr* 9 (a fungal elicitor of disease resistance) from *Cladosporium fulvum* and the *fen* gene (a homologue of the *Pto* resistance gene) from tomato (Rommens et al., 1995) among other examples.

The replacement of a nonessential viral gene by a foreign gene in order to avoid possible negative effects of increased genome size is another vector approach. This approach was the first one used. For example, gene II (470 bp) of CaMV, which encodes a protein responsible for aphid transmission of the virus, was replaced with foreign DNA without reducing infectivity (Brisson et al., 1984; De Zoeten et al., 1989). This vector was used successfully to express bacterial dihydrofolate reductase (DHFR) (Brisson et al., 1984) and human alpha-D-interferon (De Zoeten et al., 1989). Other groups have attempted to use geminiviruses, tombusviruses and several different rod-shaped viruses to express reporter genes, mostly by replacement of some or the entire coat protein gene. However, gene expression using gene replacement is rare, but some notable exceptions

include the coat protein (CP) replacement in tomato golden mosaic geminivirus (TGMV) (Hayes et al., 1989), african cassava mosaic geminivirus (ACMV) (Ward et al., 1988), and the tomato bushy stunt virus (TBSV) (Scholthof et al., 1993). In these cases only low levels of foreign gene expression were observed. Foreign gene expression by the gene replacement strategy can be adversely affected by alterations in replication, local or systemic movement, encapsidation, virus accumulation, and by recombination events inherent in some virus genomes (Chapman et al., 1992; Scholthof et al., 1993).

Potato virus X (PVX, genus Potexvirus), a single stranded RNA virus with a monopartite genome, has been developed as an efficient vector for the expression of foreign genes in plants (Oparka et al., 1995; Chapman et al., 1992; Scholthof et al., 1996). Results indicate that the coat protein of this virus plays a vital role in the key stages of the viral infection cycle. Oparka et al. (1995) have introduced the green fluorescent protein (GFP) into plant cells using PVX as a vector. The GFP was produced at high levels inside infected cells by using a duplication of the viral coat protein subgenomic RNA promoter sequence to direct transcription of mRNA encoding GFP. Hammond–Kossack et al. (1995) have reported the functional expression of a fungal avirulence gene from a modified PVX genome. Although the transgene insert in the PVX vector was not stable, the speed with which the new constructs can be generated and then assessed in a whole plant over a period of time makes the PVX vector system a highly attractive experimental too (Kearny et al., 1995). The gene of interest was coupled with a duplicated copy of the viral promoter for the coat protein mRNA before adding it to the viral genome.

English et al., (1996) demonstrated that PVX mediated expression of a foreign gene could activate a gene silencing mechanism that mediates virus resistance through co-suppression events involving RNA degradation. Culver (1996) demonstrated that transient expression (mediated by a potexvirus vector) of tobacco mosaic virus coat protein or a nontranslatable form of the gene mimicked the viral resistance by transgenic plants expressing either gene. Since PMV (potexvirus) infects papaya, it could be developed as a vector for the transient expression of foreign genes or sequences in papaya. The first step to achieve this would be the development of an infectious clone of PMV.

Papaya Mosaic Virus

Papaya mosaic virus (PMV) was first described by Conover (1962) and De Bokx (1965) as a virus causing mild mosaic symptoms in papaya. It has been reported in Florida (Conover, 1964) and Venezuela (Cook and Zettler, 1970). The virus is mechanically transmitted. No aphid transmission has been reported for this virus (Conover 1964; Zettler et al., 1968).

PMV, a member of the genus Potexvirus, was first characterized by Hiebert (1970) and further described by Purcifull and Hiebert (1971). PMV causes local lesions in *G. globosa*, a host that is used to assay infectivity. The virus is well characterized at the molecular level and the sequence is available (GenBank D13957). PMV in Florida is relatively mild in papaya with little reduction in plant growth fruit size, or yield (Conover, 1964). The virus has a limited host range outside of papaya and has no known insect vector, so environmental release of the virus vector in a crop should not present an ecological hazard.

General Characteristics of the Potexviruses

Individual potexviruses have rather limited host ranges, but collectively the potexviruses infect a wide variety of species. They are stable and transmitted mechanically. The potexviruses use subgenomic RNAs in order to translate their 3' genes. Potexviruses have a genome that consists of five open reading frames (ORFs). The first ORF encodes a protein that has domains of sequence similarity with the polymerase genes of other RNA viruses. ORFs 2, 3, and 4 are called a triple gene block and are involved in cell-to-cell movement and ORF 5 contains the coat protein. There is a polyadenylation signal at the 3' terminus. Among the plant viruses with flexuous rod-shaped particles the assembly *in vitro* of potexviruses is well documented, in particular for papaya mosaic virus. Potato virus X is the type member of the family, has a 5' m7GpppG cap structure and a poly A tail at the 3' end.

Genome Organization and Characteristics of PMV

PMV is a flexuous rod-shaped member virus. The viral genome consists of a single positive-sense RNA molecule with a 5'm^7 GpppG cap structure and a 3' poly (A) tail (AbouHaidar, 1988). It has five ORFs that encode proteins of 176 kDa, 26 kDa, 12 kDa, 7 kDa, and 23 kDa, (Sit et al., 1989). The virus RNA acts as an mRNA for the *in vitro* expression of the 176 kDa protein (Bendena et al., 1985). The 23 kDa capsid protein is expressed from a 1 kb, subgenomic RNA (Mackie et al., 1988).

In vitro assembly of PMV

Erickson and Bancroft (1978a) have described the assembly process as one dependent on RNA and coat protein interactions consisting of two major stages: helix initiation and elongation. The initiation appears to involve an interaction between the

coat protein and the 5' end of the viral RNA (Erickson and Bancroft, 1978a, 1978b; AbouHaidar and Bancroft 1978b). The elongation process occurs in a 5'-3' direction by addition of trimers and dimers until the completed rods are assembled (Erickson and Bancroft 1978b).

In Vitro Translation of Potexviruses

In vitro translation of potexviral genomic RNA in rabbit reticulocite lysate system yielded a large non-structural protein between 150-182 kDa (Bendena and Mackie, 1986). Bendena and Mackie found that this protein is translated from a cistron near the 5' end of the genomic RNA. In vitro translation of genomic RNAs of potexviruses has not resulted in high yields of the coat proteins (Atabekov and Morozov, 1979; Bendena and Mackie, 1986 Guilford and Forster, 1986).

Infectious RNA transcripts derived from PMV

Sit and AbouHaidar (1993) generated genomic length cDNAs of PMV. Infectious transcripts of PMV were produced using in vitro run-off transcription with T7 RNA polymerase. The infectivity of the transcripts was about 16% of the 'wild type' PMV. They have confirmed that an increase in the length of the poly (A) tail increases infectivity of the transcripts and the addition of non-viral nucleotides at the 3' end of these transcripts reduced infectivity but did not eliminate it.

A single amino acid deletion in the coat protein sequence was found to result in the production of smaller local lesions in *G. globosa.* Transcripts without the cap structure, although biologically active, were less infectious.

The objective of this study was to develop an infectious clone under the control of the 35S CaMV promoter that might be used to express foreign genes in papaya. The steps taken to obtain the objectives were: to (i) to construct a full-length clone of PMV, (ii) to fuse the 35S RNA promoter of CaMV to the 5' end of PMV and (iii) to determine infectivity of the clone on papaya.

The infectious clone of PMV could be used later as a vector for the PRV CP and may have several advantages over the use of transgenic plants.

CHAPTER 2
DEVELOPMENT OF A PAPAYA MOSAIC VIRUS cDNA CLONE

Introduction

Papaya mosaic virus is 6,656 nucleotides long, excluding the poly (A) tail. The virus contains five ORFs . Open reading frames 1-4, nearest to the 5' end, each overlap with adjacent ORFs and code for proteins of 176,307 Da, 26,248 Da, 11,949 Da, and 7,224 Da, respectively. The fifth ORF codes for the coat protein of 23,043 Da. A m^7GpppG cap structure is located at the 5'end (AbouHaidar and Bancroft, 1978a) and the 3' end has a poly (A) tail (AbouHaidar, 1988).

Complementary DNA to PMV RNA was synthesized by Roy et al. (1988) by reverse transcription, cloned into pUC 18 and amplified in *Escherichia coli*. Cloned DNA and cDNA were directly used as probes (biotin and ^{32} P-labelled) to detect PMV RNA in plants. Sit and AbouHaidar (1993) developed RNA transcripts from cloned cDNA of PMV and studied the effect of mutations to the capsid and polymerase proteins. This was the first report of generation of infectious RNA transcripts from cDNA of PMV. They produced infectious RNA transcripts by *in vitro* run-off transcription using T7 RNA polymerase. In this study complementary DNA of PMV was generated from RNA obtained from purified PMV.

Materials and Methods

Inoculation

Young papaya plants of the 'Solo' variety were grown from seed and kept in greenhouse reserved for healthy material until the time of inoculation. The papaya plants were mechanically inoculated. The original inoculum used was from dried leaf material of *Nicotiana benthamiana* infected with PMV (accession number PV-204) from the American Type Culture Collection (ATCC). The inoculum was prepared as follows. Dried leaf material was crushed in a mortar with a pestle and mixed with water in a 1:1 ratio (w/v). This inoculum was applied to the leaves previously dusted with carborundum by gently rubbing with a pestle. The experiment included PMV (positive) controls and healthy (negative) controls. The plants were grown in a greenhouse at about 28° C. After two weeks, the first symptoms were evident on young leaves. Leaves with typical PMV symptoms were collected after 4 weeks for extraction and purification of PMV.

Purification

PMV was purified by the method of Purcifull and Hiebert, (1971) with modifications as follows. Plant tissue was homogenized in a mixture containing one volume of 2 mM borate buffer pH 7.6 containing 0.5% sodium sulfite, 0.5 volume of butanol, and 0.5 volume of chloroform. The homogenate was centrifuged at 10,000 rpm in a GSA rotor for 15 minutes, and the supernatant was collected. The supernatant was treated with polyethylene glycol (MW 8000) at a final concentration of 5% followed by stirring for one hour at 4° C. After precipitation, the suspension was centrifuged at 10,000 rpm for five minutes, and the resulting pellet was

resuspended in 5 mM TRIS buffer, pH 8.0. The suspension was centrifuged again at 10,000 rpm using a SS 34 rotor. The yield of PMV in the supernatant was determined using a Beckman spectrophotometer at 260 nm. To determine the condition of the virus particles, a sample was analyzed by electron microscopy.

RNA Extraction

The purified virus suspension was mixed in a 1:1 ratio with RNA dissociating solution (0.2 M TRIS, 2% SDS, 2 mM Na EDTA pH 9, sterilized) and 0.01 volume of proteinase K (10 mg/ml) was added. The mixture was left at room temperature for five minutes, and 0.5 volume of phenol and 0.5 volume of chloroform:isoamyl alcohol (24:1) were added. The preparation was emulsified for five minutes and then centrifuged for five minutes at 13,000 rpm at 4° C. The aqueous phase was collected, mixed with an equal volume of chloroform: isoamyl alcohol, emulsified for five minutes, and centrifuged again at 13,000 rpm for five minutes. The aqueous phase was collected, and nucleic acids precipitated with 2.5 volumes of of 100% ethanol and 0.1 volume of 3 M NaAc . This was stored at –80° C for one hour. After precipitation, the solution was centrifuged at 13,000 rpm for 30 seconds at 4° C. The supernatant was discarded and the pellet was carefully washed twice by centrifugation with 200 µl of 70% ethanol. The alcohol was removed and the pellet was vacuum dried for six minutes and then resuspended in 50 µl of RNase free water. Recovery of the viral RNA was confirmed by electrophoresis in a 0.9% agarose gel in 1X TAE buffer at 80V for 1 hour. The concentration of the RNA was estimated to be 5µg/µl.

First Strand Synthesis

First strand cDNA was synthesized using Superscript ™ II (Gibco, BRL) for RT-PCR. Using nuclease free tubes, a 20 µl mixture was prepared as described by Life Technologies: 1 µl of oligo dT (100 µg/ µl) primer, 3 µg of RNA, and sterile distilled water to 12 µl. The mixture was heated at 70° C for 10 minutes and quickly-chilled on ice. The content of the tube was collected by brief centrifugation, followed by adding 4 µl of 5X First strand buffer, 2 µl of 1 mM DTT, and 1µl of 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH). The content was mixed and incubated at 42° C for two minutes, after which 1µl (200 units) of Superscript ™ III RNase H Reverse transcriptase was added. The mixture was incubated at 42° C for 50 minutes after which the reaction was heated at 70° C for 15 minutes for inactivation. The cDNA yield was determined by gel electrophoresis (Figure 2-1).

Preparation of cDNA of PMV

To generate the second strand and amplify the cDNA, specific primers were used. Primer EH 296 was used for the 5' end of PMV as the forward primer and primer EH 297 as the reverse primer to amplify nucleotides 1-3060 of the PMV sequence (Table 2-1). Two other primers were designed to amplify the remaining sequence from nucleotide 2940, primer EH 295 as the forward primer, and primer EH 258-259-260, the oligo dT complementary to the poly A tail of PMV as the reverse primer.

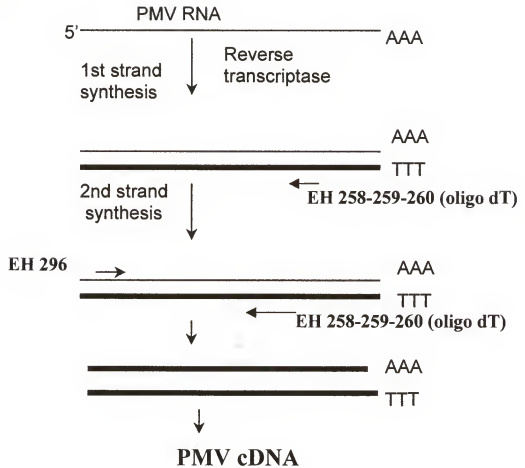

Fig. 2-1. Schematic representation of first strand synthesis.

Table 2-1. Sequence of specific oligonucleotides used for PCR-amplification of 6.7 kb cDNA of PMV. Modifications to the general conditions described are indicated.

PRIMER NAME	PRIMER DESCRIPTION	OLIGONUCLEOTIDE SEQUENCE	RESTRICTION ENZYME USED
EH 296	From 5'end of PMV Forward	GGAAAAGAAACACAAAGCAAAGC	Bgl II
EH 297	From nt 3060 of PMV Reverse	TAAAGCGAGGGCACTAGCATG	Bgl II
EH 295	From nt 2940 of PMV Forward	CATCAATGCCACACACAG	Bgl II
EH 258,259,260	From poly A tract of PMV Reverse	TTTTTTTTTTTTTTTTTTT(A,G,C)	none

Each 50 µl of Long Template-PCR reaction contained 25 µl of Master Mix I and 25 µl of Master Mix II. Master Mix I consisted of 9 µl of RNase free water, 10 mM dNTPs, 1 µl of each primer 100 (pmol) and 1µl of first strand. Each 25 µl of Master Mix II reaction contained 16.25 µl of nuclease free water, 5 µl of 10X buffer 3 (Boehringer/Mannheim), 3µl of 25 mM $MgCl_2$ and 0.75 µl of enzyme mix (Taq/Pwo). The two mixes were combined in a thin–walled PCR tube and overlayed with 30 µl of mineral oil. The reactions were amplified using a thermocycler (Biometra Inc., Tampa, Fl) for 1 cycle at 94° C for two minutes; 10 cycles composed of 10 seconds for denaturation at 94° C, 30 seconds for annealing time at 44° C and 4 minutes for extension at 68° C. This was followed by another 20 cycles in which the elongation step was increased from 4-7 minutes in 20-second increments after each successive cycle. The reaction ended with an elongation cycle at 68° C for 7 minutes.

To confirm that the complete genome was amplified from the cDNA, the different sections were amplified by PCR using first, primers EH 296 (forward) and EH 297 (reverse), to amplify a 3,060 bp fragment (from nt 1-3060 of PMV); second, primers EH 295 (forward) and EH 258-260 (reverse) to amplify a 3,716 bp fragment (from nt 2940-poly A tract of PMV). Finally, to obtain a 6,656 bp fragment that spanned the length of the PMV sequence, primers EH 296 (forward) and EH 258-260 (reverse) were used. This product included the complete sequence of PMV from the 5' end of PMV to the poly A tract. Aliquots of 3 µl of the products were mixed with 1 µl of tracking dye and added to wells of a 0.6% agarose gel in 1X TAE (40 mM TRIS–acetate, 1 mM EDTA, pH 8.2) to which ethidium bromide (1 mg/ml) was

added. Agarose gel electrophoresis was performed at 80 V for 1 hour, visualized on a transilluminator and photographed. PCR products were directly cloned into pGEM T Easy Vector (Promega Co., Madison, WI) and transformed into *Escherichia coli* DH 5 α cells. Two clones that contained appropriately sized inserts were selected and the ends sequenced. DNA samples were sequenced by the DNA sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR).

Analysis of PMV Clones

Plasmids were purified according to a miniprep procedure described by Qiagen Inc. (Chadsworth, CA). Up to 1.5 ml of cell culture was collected by centrifugation in a microcentrifuge tube at 12,000 rpm for 1 minute at 4° C. The cells were resuspended in 250 µl of P 1 buffer (50 mM TRIS HCl, pH 8.0, 10 mM EDTA, RNase A at 100 µg/µl). After incubation at room temperature for 5 minutes, 250 µl of P 2 solution (200 mM NaOH, 1% SDS) were added and the contents were mixed gently by inversion. After additional 350 µl of P 3 solution (3 M potassium acetate, pH 5.5), the mixture was centrifuged at 12,000 rpm for 5 minutes, the supernatant was transferred to a fresh tube, and one volume of 100 % isopropanol was added. The mixture was then centrifuged at 12,000 rpm for 5 min. The pellet was resuspended in 25 µl of sterile water, and the material screened by *Not* I (Promega) digestion for clones of the correct size. Aliquots of 5 µl of the products were mixed with 1 µl of tracking dye and added to wells of a 0.6% agarose gel in 1X TAE (40 mM TRIS –acetate, 1 mM EDTA pH 8.2) plus 1 µl of ethidium bromide (1mg/ml).

Agarose gel electrophoresis was performed at 80 V for 1 hour. The results were visualized on a transilluminator and photographed.

Results

Purification of PMV and Isolation of Viral RNA

The PMV virions were purified successfully from papaya plants inoculated with PMV. The $A_{260/280}$ ratio was 1.39. The estimated virus yield was 5 mg/100 g leaf tissue. Electron microscopic examination of the virus preparation revealed numerous particles.

Viral RNA was readily extracted in RNA dissociating solution and proteinase K followed by phenol/chloroform extraction. This required a minimum time to process, and reduced the possibility of degradation and possible damage. The yield of viral RNA extracted was estimated to be 5 μg/μl (Figure 2-2.).

cDNA of PMV

In order to generate full-length cDNA clones of PMV, two separate approaches were taken. The first one involved the ligation of two subclones of PMV generated from two segments of PMV that were obtained from PCR: a fragment from the 5' end to nucleotide 3060 and another fragment from nucleotide 2940 to the poly A tract in order to produce a full size clone. This approach did not yield any clones of interest and correct ligation of the two large fragments was not achieved.

The second approach involved synthesis of full-length cDNA using Superscript™II. This method yielded full-length first strand cDNA.

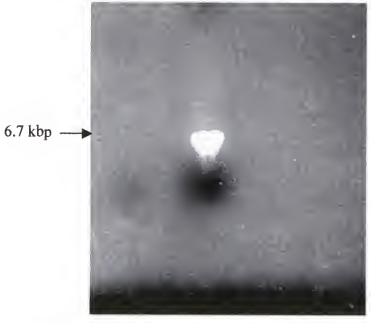

Figure. 2-2. Agarose gel electrophoresis of PMV RNA isolated from purified PMV virions by phenol/chloroform extraction. The viral RNA was separated in a 0.9% agarose gel, stained with ethidium bromide and visualized by UV transillumination.

Full-length cDNA (6.7 kbp) was obtained after first strand synthesis and long template PCR using primers EH 296 and EH 258-260, that are specific for the 5' and 3' ends respectively. PCR products obtained consisted of segments of the PMV genome includingnucleotide 1-3060 (3,060 nt in length), 2940-oligo dT (3,716 nt in length) and one spanning the complete sequence of PMV, 1-oligo dT (6,656 nt). The products were estimated to be 3.0 kbp, 3.7 kbp, and 6.7 kbp (Fig. 2-3).

The 6.7 kbp cDNA was cloned into the pGEM T Easy (Promega) plasmid yielding several promising clones. After plasmid extraction, the different clones were cut with Not I to release the insert from the plasmid. Two plasmids contained inserts of interest of about 6.7 kbp, corresponding in size to the full–length PMV and were selected for further study (Fig. 2-4). This attempt was successful and the cDNA's obtained contained the complete sequence needed to continue the work, thus the alternate approach of obtaining the clone by ligating the two segments of PMV that was being worked at simultaneously was not pursued any further.

Sequencing

Sequence analysis of the two clones indicated that the clone designated as # 10 had the same sequence as PMV (100% similarity), whereas the clone 190 had a section missing at the 3' end (Figures 2-5. and 2-6.).

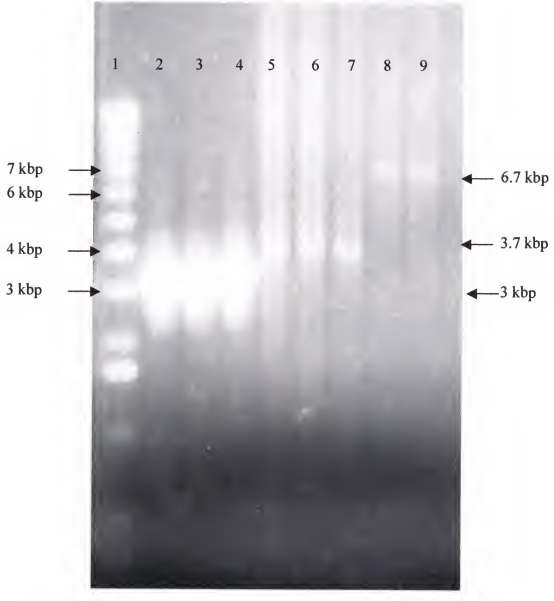

Figure 2-3. PCR products of PMV amplified with different primer combinations from the first strand and separated by agarose gel electrophoresis. Lane 1, 1 kbp DNA ladder; lanes 2-4, 3.0 kbp product; lanes 5-7, 3.7 kbp product; lanes 8-9, 6.7 kbp product.

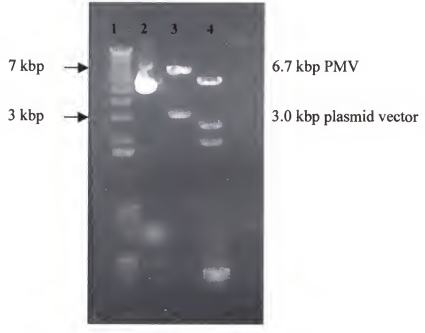

Figure 2-4. Cut and uncut plasmid extracted from colony containing full-length PMV insert separated by agarose gel electrophoresis. Lane 1, 1kbp DNA ladder; lane 2, uncut plasmid; lane 3, plasmid cut with *Not* I; lane 4, plasmid cut with *Pst* I.

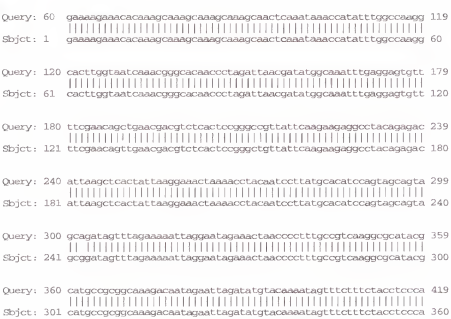

Figure 2-5. Similarity of the nucleotide sequence of the 5' terminal region of the PMV clone # 10 with a previously described sequence of the region.

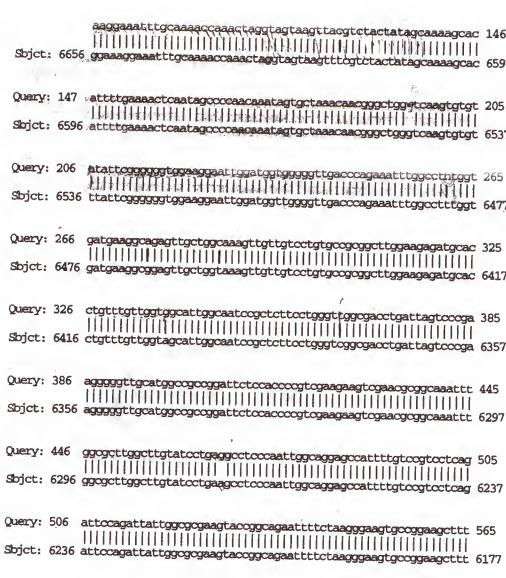

Fig. 2-6. Similarity of the nucleotide sequence of the 3' terminal region of the PMV clone # 10 with a previously described sequence of the region.

Discussion

Two different approaches were taken in order to obtain full-length cDNA clones of PMV. The first one involved attempts to ligate the 3.0 kbp and 3.7 kbp subclones of PMV obtained from PCR in order to produce a full size clone. This approach did not yield any clones of interest and correct ligation of the two large fragments was not achieved.

The second approach involved synthesis of full-length cDNA using Superscript™II. This method yielded full-length first strand cDNA.

During the process of clone selection it was observed that clones that were selected as containing inserts would lose the inserts upon overnight cultivation of the bacteria (DH 5α). This problem was solved by using ultracompetent cells such as Sure™ 2 (Stratagene, La Jolla, CA). These cells are engineered so that they do not reject inserts of large sizes.

A 6.7 kbp clone of PMV was obtained which contained the insert of the estimated length of the PMV genome. Of the clones obtained, two were selected and sequenced. One was missing a section at the 3' end. This eliminated this clone as a possible candidate for further work. The second clone sequenced, contained the complete sequence and had a high degree of similarity with PMV. This clone was chosen to continue the work of making an infectious clone.

CHAPTER 3
ADDITION OF THE CAULIFLOWER MOSAIC VIRUS 35S PROMOTER TO PAPAYA MOSAIC VIRUS

Introduction

Infectious transcripts of PMV under the control of the T7 promoter were synthesized *in vitro* by AbouHaidar and Sit (1993). Their results indicate that the infectivity of the transcripts was 16% of that of the native PMV RNA and that increasing the length of the poly (A) tail from A_{24} to A_{71} increased infectivity to 42 %. Uncapped transcripts were much less infectious than capped transcripts. They introduced mutations into the polymerase and CP coding regions but most transcripts appeared were found to be biologically inactive. A single amino acid deletion in the CP resulted in smaller local lesions from which viral particles could not be observed. The authors suggested that the virus might exist as a naked RNA species within the host (AbouHaidar and Sit, 1993).

The 35S CaMV promoter has been used to obtain infectious clones of several plant viruses, some of these include brome mosaic bromovirus (Mori et al., 1991), BMYVV furovirus (Commandeur et al., 1991), plum pox potyvirus (Maiss et al., 1992), tomato aspermy cucumovirus (Shi, B-J., et al., 1997).

In the present study, clones of PMV were modified by adding the 538 bp 35S promoter from the plasmid pCambia 1302 using a series of overlapping PCR steps.

The objective was to obtain a construct that was infectious after mechanical or ballistic inoculation.

Materials and Methods

Addition of 35S CaMV promoter from pCambia to PMV

In order to add the 35S promoter from pCambia 1302 to PMV clone 10, one set of primers primers were designed containing the 5' end sequence of the 35 S promoter of pCambia and the 3' end primer contained the end sequence of the 35 S promoter plus the first bases of the 5' end of PMV. This resulted in a PCR product that contained the 35 S promoter and the first bases of the 5' end of PMV. Also, PMV was amplified from the 5' end to the 3' end using a primer for the 3' end that added 20 a's to the poly A tail. Using these two products obtained, each containing overlapping regions (the 3' end of the 35 S promoter and the 5' end of PMV) both were assembled together through a series of overlapping PCR steps to form one product: the PMV with the 35 S CaMV promoter added directly to the 5' end (Figure 3-1). The primers designed for the overlapping PCR reactions are described (Table 3-1.) Primer A (EH 333), a positive sense primer with 4 random initial bases (for restriction endonucleases cutting purposes), an *Xba*I site and the first 17 bases of the 35S promoter of pCambia. Primer B (EH 334) was a negative sense primer with the last 18 bases of the 35S CaMV promoter from pCambia plus the first 20 bases of PMV. Primer C (EH 296) was a positive sense primer with the first 20 bases of PMV. Primer D (EH 336) was a negative sense primer with the last 20 bases of

PMV, 20 T's, and a *Kpn*I (*Asp 718*) site.

Sequence of PCR Reactions

The first step was to amplify a region of pCambia 1302 containing the entire CaMV 35S promoter by using primers A (forward) and B (reverse). The cells containing the pCambia were stored at –80° C in LB freeze medium. An aliquot of 100 µl of the cell suspension was added to 3 ml LB broth with kanamycin (25 mg/ml) and grown overnight at 37° C at 225 rpm. An aliquot of 100 µl was retrieved and added to 100 µl of RNase free water. The contents of the tube were frozen for 5 minutes, boiled for 5 minutes and then diluted to a concentration of 1:100. The plasmid product was purified using Qiagen columns (Qiagen, Valencia, CA), eluted in 50 µl of RNase free water and stored at –20° C. This product was then used as template for the first PCR reaction to obtain the 35S promoter from the pCambia (Figure 3-1.).

The PCR reaction after standardization was conducted in thin wall tubes and was composed of 20 µl of Supermix (Promega), 1 µl of 10 pmol primer A, 1 µl of 10 pmol primer B and 2 µl of the 1:100 pCambia 1302 as described (Table 3-1.). The thermocycler program was one cycle at 94° C for 2 minutes, ten cycles of denaturation at 94° C for 10 seconds, annealing at 44° C for 30 seconds, and elongation at 68° C for 4 minutes; this was followed by 20 cycles of denaturation at 94° C for 10 seconds, annealing at 44° C for 30 seconds and elongation at 68° C for 4 minutes. This was followed by another 20 cycles in which the elongation step was increased from 4-7 minutes in 20-second increments after each successive cycle. The reaction ended with an elongation cycle at 68° C for 7 minutes.

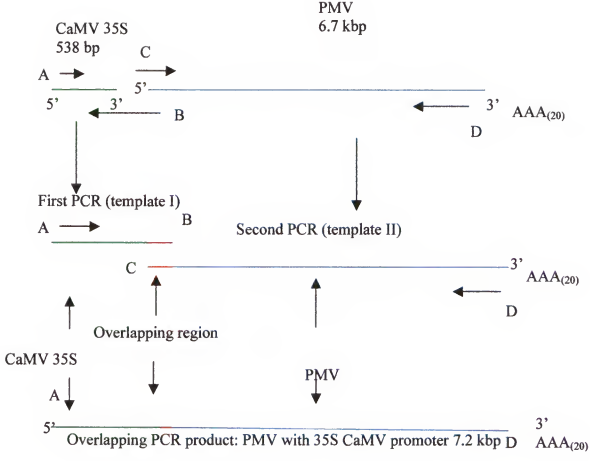

Figure 3-1. Schematic representation of the sequence of PCR reactions performed to attach the 35S CaMV promoter to the 5' end of PMV. Green line represents pCambia, blue line represents PMV, small red line represents overlapping region after first PCR.

Table 3-1. Sequence of specific oligonucleotides used for overlapping PCR for addition of the 35S CaMV promoter.

PRIMER NAME	DESCRIPTION	OLIGONUCLEOTIDE SEQUENCE (5'-3')	RESTRICTION ENZYME SITE
Primer A Forward EH 333	AGCC+*Xba*I site + first 17 bases of 35SCaMVpromoter	AGCCTCTAGACATGGAGTCAAAGATTCAAATAG	*Xba*I
Primer B Reverse EH 334	Last bases of 35S CaMV promoter	TGCTTTGCTTTGTGTTTCTTTTCCAGTCCCCCGTGTTCTCTC	none
Primer C Forward EH 296	First 23 bases of 5' end of PMV	GGAAAAGAAACACAAAGCAAAGC	none
Primer D Reverse EH 336	Last 20 bases of PMV + 20 T's + *Kpn*I site	GGCTGGTACCTTTTTTTTTTTTTTTTTTTTTGGAAAGGAAATTTGCA AAAC	*Kpn*I

The product contained 4 random bases, a *Xba*I site, the 35S promoter of pCambia and the first 20 bases of PMV, resulting in a product of 568 bp. The PCR product was purified using the PCR purification column kit of Qiagen (Valencia, CA). The DNA was eluted in 50 µl of RNase free water and stored at –20° C. Five microliters of the resulting PCR product was analyzed by electrophoresis in a 0.8 % agarose gel in 1X TAE buffer (containing 1mg/ml of ethidium bromide stain) at 80 V for 1 hour.

The second PCR was conducted with primers C and D in order to obtain a region of the PMV clone from the first 20 bases to the 3' end with the addition of a poly (A) tail and a terminal *Kpn*I site. The resulting PCR product totaled 6,692 bp. The reaction mixture included: 20 µl of Supermix (Promega), 1 µl of 10 mmol primer EH 296, 1µl of 10 mmol of primer EH 336, and 1 µl of a 1:10 dilution of clone 10 as template. The thermocycler program was the same as described for the first PCR. The PCR product was purified using Qiagen columns (Qiagen), eluted in 50 µl of RNase free water and stored at – 20°C and run in a 0.6% agarose gel 1X TAE buffer with ethidium bromide (1 mg/ml) at 80 V for 1 hour. The products of the first two reactions had an overlap of 20 bases, and were combined for the third PCR reaction using primers A and D.

The overlapping PCR was standardized according to the Long Template PCR System described by Boehringer/Mannheim. The Master Mix I contained 11µl of RNase free water, 2.5 µl of 10 mM dNTPs, 1 µl of the forward primer A (10 pmol), 1 µl of the primer D (10pmol), 1 µl of the product of the first PCR diluted 1:10 as one template, plus 1 µl of the product of the second PCR diluted 1:10 dilution as the second template. The

Master Mix II contained (per reaction) 17.75 µl RNase free water, 5 µl 10X buffer 3, 1.5 µl of 25 mM $MgCl_2$, and 0.75 µl of enzyme mix (Taq/Pwo). The complete Master Mix I and Master Mix II were mixed together in thin-wall tubes, and covered with mineral oil. The program cycle was the same as described for the first and second PCR . The product (5 µl) was examined by electrophoresis as described previously.

Ligation of Construct to Plasmid Vector

The 7.2 kbp product was ligated into the pGEM T Easy (Promega, Madison, WI) following protocols described in the technical manual for this vector. A reaction of a 1:1 ratio of insert to vector was used for the ligation. The reaction included 1 µl of 10X buffer, 50 ng of vector, 50 ng PCR product, and 1 µl of T4 DNA ligase to a total volume of 10 µl. The ingredients were mixed by pipetting and incubated overnight at 4° C.

Transformation

Transformation was accomplished by using Sure™ 2 ultracompetent cells (Stratagene Cloning Systems, La Jolla, California). The cells were thawed on ice, gently mixed by hand and an aliquot of 100 µl was added to pre-chilled tubes (Starsted Newton, NC). Two microliters of β-mercaptoethanol (Stratagene) were added to the aliquots to give a final concentration of 25 mM in each tube. The contents of the tube was swirled and incubated on ice for 10 minutes. The DNA (50 ng) from the ligation reaction was added to the cells and swirled gently, and incubated for 30 minutes. The cells were heat pulsed in a 42° C water bath for exactly 30 seconds and incubated quickly on ice for two minutes. NYZ^+ broth (900 µl) preheated at 42° C was added to the tubes and the mixture

was incubated at 37° C for one hour with shaking at 225 rpm. The cells were plated on LB agar with 100 µl of 10 mM IPTG (isopropyl-B-D-thio-galactopyranoside) and 100 µl of 2% X-gal (5-bromo-4-chloro-3-indoyl-B-D-galactopyranoside). The plates were incubated overnight at 37° C.

Screening Colonies for Inserts

White colonies were selected and screened for inserts by using PCR. Samples from groups of ten colonies were pooled by collecting a small sample of each colony growing on LB medium by means of a toothpick and shaking it into the PCR mixture. Using primers specific for the CP of PMV a reaction mixture was prepared that consisted of 22 µl Supermix, 1 µl of each of the primers and the pooled sample from 10 colonies. The preparations were subjected to 30 cycles denaturation at 94° C for 1 minute, annealing at 59° C for 1.5 minutes, and extension at 72° C for 1 minute followed by a final extension of 72° C for 10 minutes. The resulting PCR products were examined by electrophoresis as described above. The size of the expected product was 819 bp. The sets of 10 colonies that were positive for the 819 bp band were selected, the colonies cultured individually overnight on LB broth and 1 µl of suspension was used for a second individual PCR reaction with primers A and D, specific for the 7.2 kbp product. The PCR reaction was established as follows: 20 µl Supermix, 1 µl of primer A and D each and 1µl of cell suspension from overnight cultures for Master Mix I. Master Mix II was established following protocols in the Long Template PCR System of

Boehringer/Mannheim using buffer system 3. The cycling program used was the one previously described for long template PCR.

Selection of Colony With Insert

Colonies containing clones for which a correctly sized amplification product was detected by PCR were selected for the first examination. After overnight culture and plasmid extraction, the cloned insert was cut into fragments using *Bgl*II (Promega) that cuts PMV once at 2966-67, *Not*I (Promega) that cuts the insert out of the plasmid and *Sal*I (Promega) that cuts only the plasmid at one site. The colony selected contained the insert of the correct size and was stored in LB medium at – 80° C.

Results

First PCR Step

An amplification product was obtained after the first PCR step using primers specific for the 35S CaMV promoter from pCambia 1302. The PCR fragment obtained was 548 bp in length and corresponded to the expected size of the 35S CaMV promoter with the *Xba*I site and the 4 random bases. The product was then used as template for the overlapping PCR reaction.

Second PCR Step

In the second PCR reaction, a product was obtained that contained the cDNA of PMV with a poly (A) tail of 20 T's followed by a *Kpn*I site. The product totaled 6,692 bp.

Overlapping PCR Resulting in Construct Assembly

A 7.2 kbp product was obtained as a result of the overlapping PCR by using the 548 bp and 6.7kbp products from the first and second PCR steps as templates (Figure 3-2.). The 7.2 kbp product contained AGCC (4 bp) + *Xba*I site (6 bp) + CaMV 35S promoter (538 bp) + PMV (6,656 bp) + poly $(A)_{20}$ tail + *Kpn* I site (10 bp) (Figure 3-2.)

Cloning of the 7.2 kbp fragment

The 7.2 kbp product was ligated successfully into the pGEM T Easy vector. Numerous attempts to transform cells of *E. coli* DH 5 α with the vector containing the 7.2 kbp insert were unsuccessful. When using DH 5 α, several clones containing the putative fragment were obtained, but repeatedly lost when further bacterial cultures were established as required for plasmid purification.

The recombinant vector was transformed into Sure ™ 2 ultracompetent cells (Stratagene, La Jolla, CA). These cells are designed to accept and keep larger fragments and do not contain any correction mechanisms that would allow the loss of the insert of interest due to size. Among the 182 white colonies screened after transformation, and screening, only one, designated as 124, was determined by PCR to contain both the coat protein of PMV as well as the complete 7.2 kbp insert. Other white colonies contained no inserts at all or inserts smaller than the 7.2 kbp fragment expected. Restriction digests produced patterns expected for each enzyme. The plasmid containing the insert was cut with *Bgl*II resulting in a 10.2 kbp product: 3 kbp of plasmid, and 7.2 kbp of insert. When

the plasmid was cut with *Not*I, which cuts the insert out of the plasmid, two products were obtained, the 3 kbp plasmid and the 7.2 kbp insert. When the plasmid was cut with *Sal*I, which cuts the plasmid vector once, a 10.2 kbp product was obtained (Figure 3-3.).

Discussion

The first step in the process of making an infectious cDNA clone of PMV was to add a promoter. Using PCR, the 35S promoter was successfully amplified from the vector pCambia. Next, a copy of PMV was amplified starting from the 5' end and a series of 20 T's were added to code for a poly (A) tail at the 3' end. A longer poly (A) tract promotes an increase in infectivity (Sit and Abouhaidar, 1992). Finally, a 7.2 kbp product was produced by overlapping PCR that contained all the elements required for the construct. The results of the restriction digests corroborated the previous results and indicated that clone 124 contained a 7.2 kbp fragment including the 35S CaMV promoter directly attached to the 5' end of PMV as well as a 20 bp poly (A) tail with a *Kpn*I site at the 3' end.

For the development of RNA viruses as gene vectors, it is crucial to obtain infectious clones of their genomes (Goldbach and Hohn, 1997). Fusing a cDNA promoter to a cDNA clone of the virus is an approach that results in a more efficient production of virus progeny as was first reported for both brome mosaic virus (BMV) (Mori et al., 1991), and beet necrotic yellow vein virus (BNYVV) (Commandeur et al.,

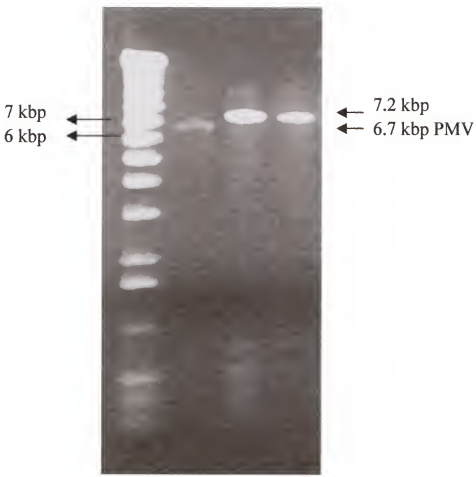

Figure 3-2. PCR products of PMV cDNA and PMV cDNA with the fused 35S CaMV promoter. Lane 1 shows 1 kbp DNA ladder; lane 2, PMV cDNA 6.7 kbp product; lanes 3-4 PMV with the 35S CaMV promoter added, 7.2 kbp product.

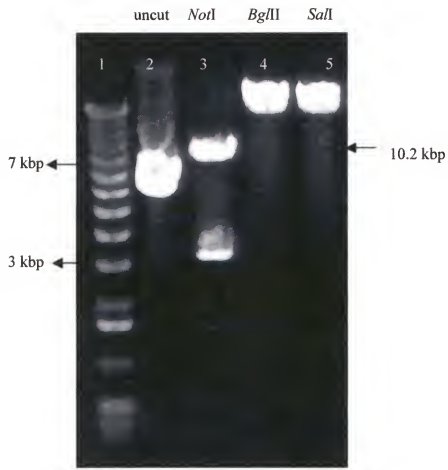

Figure 3-3. Restriction digests of PMV clone # 124.
Lane 1, 1 kbp DNA ladder; lane 2 uncut plasmid; lane 3, plasmid cut with *Not*I; lane 4, plasmid cut with *Bgl*II; lane 5, plasmid cut with *Sal*I. *Not*I releases the insert of 7.2 kbp from the plasmid. *Bgl*II and *Sal*I cut the insert once both lanes 4 and 5 showing linearized insert with plasmid together as one band.

1991), and more recently for another seven viruses belonging to different taxonomic groups (Boyer and Haenni, 1994).

The overlapping PCR was used as a strategy to eliminate any nonviral nucleotides between the promoter and the 5'end of the viral transcripts, which are known to reduce infectivity (Boyer and Haenni, 1994). Several other factors may have a dramatic influence on the infectivity of a clone in addition to the sequence of the 5' and 3'ends (number and sequence of nonviral nucleotides), including the presence of a cap structure at the 5' end or a poly (A) tail at the 3' end. As a general rule, 5' extensions of non-viral nucleotides at the ends of the viral transcript substantially decrease or abolish infectivity, whereas 3'end extensions are more easily tolerated (Goldbach and Hohn, 1997).

For the development of transformants that retain inserts of large sizes as the ones used in this study, it was critical to use cells that were designed to accept large inserts. Many transformations were done using DH5 α cells. The transformants obtained would be screened for the insert expected, and the ones containing the inserts were selected for further culture and plasmid extraction. Invariably and repeatedly the fragments were lost presumably due to recombination and correction mechanisms inherent in the bacteria. These cells that did not allow large inserts. The problem was solved by using ultracompetent cells Sure 2 (Stratagene), cells designed to accept large inserts. The colonies of these cells containing the inserts could be re-cultured and the plasmid extracted without the loss of the insert of interest.

The construct obtained is predicted to have no nonviral nucleotides at the 5' end. The region in which both parts were fused was not sequenced to determine if precise fusion had been achieved.

The construct obtained in this study contained a CaMV 35S promoter sequence directly adjacent to the 5'end of a cDNA of PMV and should result in a RNA transcript with no additional bases at the 5'end the PMV sequence with a poly (A) tract of 20 A's at the end.

CHAPTER 4
TESTING OF THE CONSTRUCT OF PAPAYA MOSAIC VIRUS WITH 35S PROMOTER FOR INFECTIVITY

Introduction

The use of plant viruses as vectors to introduce and express non-viral genes in plants is well documented (Donson, et al., 1991; Chapman et al., 1992; Goldbach and Hohn, 1997; Dolja et al., 1998). This does not lead to permanent incorporation of the foreign gene into the plant genome, however, gene expression and virus multiplication can continue indefinitely. The *in vitro* transcription method is the most common procedure employed for reverse genetics studies on cloned plant RNA virus genomes. Recently, ligation of the 35S promoter of CaMV upstream from the full-length cDNA of the virus, followed by direct inoculation with the construct is increasingly being used to infect plants with cloned RNA viruses (Scholthof et al., 1996). The approach results in an efficient production of progeny virus (Mori et al., 1991; Commandeur et al., 1991; Shi et al., 1997). The infectivity of several 35S cDNA clones by manual inoculation onto intact plants has been reported for, pea early browning virus (McFarlane et al., 1992), TMV-L (Weber et al., 1992), plum pox virus (Maiss et al., 1992), cowpea mosaic virus (Dessens and Lomonossoff, 1993), alfalfa mosaic virus (Neeleman et al., 1993), and cucumber mosaic virus (Ding et al., 1995). Shi et al. (1997) obtained infectivity for infectious cDNA clones of tomato aspermy virus by using a duplicated 35S CaMV promoter. The system with the 35S CaMV promoter

offers several advantages, including easy preparation of plasmid inocula and plant infection by conventional mechanical inoculation, when compared to other strategies that have also been used to obtain progeny virus from cDNA clones such as infectious RNAs transcribed *in vitro* (Boyer and Haenni, 1994) and particle bombardment of plants with cDNA clones (Gal-On et al., 1995; Fakhfakh et al., 1996).

In this chapter, I describe the results of different strategies used to inoculate plants with PMV clone # 124 and the results on infectivity on *Carica papaya*, *Gomphrena globosa* and *Nicotiana benthamiana* plants.

Materials and Methods

Ballistic Inoculation

Plants were inoculated using particle bombardment with a helium flow apparatus made at the University of Florida. The inoculation mixture consisted of 100 µM spermidine, 2.5 M CaCl, tungsten particles (sterilized, pre-washed in 95% ethanol and resuspended in water at 100 µg/µl) and DNA to a final concentration of 1 µg/µl. PMV clone #124 in pGEM T Easy plasmid vector was used for all the experiments described in this chapter. Plants were inoculated with the PMV clone as the uncut plasmid, the plasmid cut with *Not*I (this releases the insert from the plasmid), and the plasmid cut with *Kpn*I (this cuts at the 3' end of the construct). Positive and negative controls were inoculated with PMV and water, respectively. For inoculation, 3 µl were placed in the center of a plastic Gelmann 3mm filter holder (Gelmann Sciences Inc., Ann Arbor, MI). The filter unit was attached to the helium flow apparatus and helium (650 kPa) was released through the filter unit for 1 second in the bombardment chamber under vacuum (25 psi). Preliminary experiments showed that ballistic

inoculation caused injury of small plants, particularly *N. benthamiana* caused injury. The optimal distance between the gun and the leaf was adjusted to approximately 6 cm from the source in order to limit tissue damage. This was established by doing mock-inoculations of the three kinds of plants to be used for the experiments at 2, 4, 6, 8, and 10 cm from the source. The damage to the plants and the effectiveness of the inoculations was recorded.

Plants of *C. papaya*, *N. benthamiana* and *G. globosa* were inoculated using four plants per treatment. After inoculation the plants were planted in pots, transferred to a greenhouse, and kept covered for the first 3 days.

Mechanical Inoculation

Plants were mechanically inoculated with inoculum prepared as previously described for the ballistic inoculations. A final DNA concentration of 1 μg/μl was used. Leaves were dusted with carborundum, and the inoculum was rubbed gently on the leaf surface with a pestle. The leaves were subsequently rinsed with water. PMV inoculated and healthy controls were kept. Four young plants per treatment of *C. papaya*, *N. benthamiana* and *G. globosa* were inoculated. After inoculation the plants were planted in pots and transferred to the greenhouse.

Ballistic Inoculation and Mechanical Inoculations: Light Versus Dark

A second ballistic and mechanical inoculation experiment was performed as previously described but only one plant of each species (*C. papaya*, *N. benthamiana* and *G. globosa*) was inoculated per treatment. One group of plants was kept in the greenhouse under normal day/night conditions prior to inoculation, while the other set of plants was placed in the dark for a period of 24 hours prior to inoculation.

The Effect of Inoculum Concentration on Infectivity of PMV Infectious Clone

Young papaya plants were mechanically inoculated with PMV construct as described previously but at concentrations of 1 µg/µl, 2 µg/µl and 3 µg/µl of uncut plasmid and with construct cut with *Kpn*I. One set of plants was kept in the dark for 24 hours at 28° C prior to inoculation, and the other was kept under normal light/day conditions. Each treatment consisted of 10 plants. The leaves were dusted with carborundum and a drop of the inoculum at the proper concentration was gently rubbed with a gloved finger. The plants were rinsed after inoculation and kept in the growth room for one day under normal day/night cycle. The next day, the plants were transferred to the greenhouse. Healthy (10 plants) and PMV inoculated (8 plants) controls were kept for each set.

Tissue Blots

Detection of PMV was done using the Westernlight™ Chemiluminescent Detection System (Tropix, Inc., Bedford, MA). Rolled leaves of infected and healthy *C. papaya*, *G. globosa* and *N. benthamiana* plants were cut transversely and immediately imprinted on a nitrocellulose membrane. In the case of local lesions, the lesion was excised and then squashed on the nitrocellulose membrane (Transblot transfer medium, Bio-Rad, Hercules, CA) by using a sterile pestle. Following transfer, the membrane was washed in PBS (58 mM Na_2HPO_4, 17 mM NaH_2PO_4 . H_2O, 68 mM NaCl in H_2O), and blocked in blocking buffer (0.2 g I-Block, 1X PBS 0.01% Tween-20) for 30 minutes at room temperature. The membrane was immersed in wash buffer (1X PBS containing 0.1 % Tween-20) and probed with PMV antiserum (AGDIA Inc., Elkhardt, IN) diluted to 1:200 in blocking buffer. The membranes were then

incubated in anti-rabbit-alkaline phosphatase conjugate (Sigma Chemical Co. St. Louis, MO) diluted to 1:30,000 for 30 minutes at room temperature. After washing, the nitrocellulose membrane was incubated 5 minutes in CSPD Ready to use substrate containing 1:20 Nitro-Block (150-µl Nitro-Block + 3 ml CSPD Ready-to-Use substrate). The excess CSPD was drained and the membrane placed in a development folder and exposed to film.

Electron Microscopy

Leaf dips were prepared by cutting the tissue in 5 mM potassium phosphate buffer pH 7.0, and stained with 2% uranyl acetate. The grids were viewed on a Hitachi H-600 transmission electron microscope at 75 KV.

Enzyme-Linked Immunosorbent Assay (ELISA)

For indirect ELISA tests, samples were ground in coating buffer (12 mM sodium bicarbonate, 35 mM sodium carbonate, pH 9.6) at a ratio of 1 to 10 and added to wells of Type I polystyrene microelisa plates (Dynatech Labs, Inc., Chantilly, VA). For each well, 100 µl of sample was added. The plates were incubated at 37° C for 1 hour and rinsed with three 5-minute washes in PBS, containing 0.05% Tween–20 (PBST). PMV antibody was diluted as recommended by AGDIA (Eklhardt, IN) to 1:200 in enzyme–conjugate buffer (PBST, containing 2.0% polyvinylpyrrolidone and 0.02 % ovalbumin), and 100 µl were added to the each well and incubated at 37^0 C for 1 hour. The plates were rinsed four times as before. One hundred microliters of 2 µg/ml antirabbit IgG diluted 1:30,000 was then added to appropriate wells. The plates were incubated for 1 hour at 37^0 C and washed 3 times in PBST. One hundred microliters of substrate (ρ-nitrophenyl disodium phosphate, 1 mg/ml, Sigma Chemical

Co. (St. Louis, MO) in substrate buffer at pH 9.8 (9.7% diethanolamine, Fisher Scientific, Fair Lawn, NJ) was added to the plates and incubated at room temperature. Absorbance readings (405nm) were taken on a Bio-Tek automated microplate reader (model EL 309 Bio-Tek Instruments Inc., Winooski, VT) at fifteen-minute intervals for 1 hour.

Results

Ballistic Inoculation

Three plants of *G. globosa* and one of papaya showed PMV symptoms after ballistic inoculation, whereas none of the *N. benthamiana* plants developed symptoms (Figure 4-1.), (Table 4-1.). Plants inoculated with the construct showed fewer symptoms and the symptoms took longer to develop when compared to the PMV controls inoculated.

A few local lesions were observed in *G. globosa* when inoculated with the uncut construct, with the construct cut with *Not*I, and with the construct cut with *Kpn*I. In each case, one of each of the four plants had local lesions (Table 4-1.). All four controls inoculated with PMV were positive. The four healthy controls had no symptoms. Symptom appearance took 7 days for the PMV control whereas for the construct small local lesions were visible after 9 days. There was also a difference in the size of the local lesions. Initially, the ones inoculated with the construct were smaller after the same number of days as compared to the PMV positive control.

As time progressed these differences disappeared. In the case of papaya only one plant developed symptoms about 5 weeks after inoculation. The plant that showed

symptoms was one of four inoculated with the uncut construct. The symptoms were not systemic but were restricted only to a section of one of the lobes of a papaya leaf that was inoculated (Figure 4-2.). PMV controls showed systemic symptoms after two weeks.

Indexing

After ballistic inoculation, the three plants of *G. globosa* with local lesions were indexed with the chemiluminescent detection system and tested weakly positive. Healthy controls were negative; PMV controls exhibited a strong positive reaction (Figure 4-3.). The only papaya plant with symptoms also had a strong positive reaction in the tissue blot when compared to that of the healthy control. The PMV control had a very strong positive reaction (Figure 4-3.) (Table 4-1.).Particles were seen in leaf dip preparations of two of the three plants of *G. globosa* that had local lesions (Figure 4-4.).

The papaya plant that showed symptoms after 45 days was also indexed. Particles were seen only from the area of the papaya leaf that had symptoms. No particles were found in the same leaf in symptomless areas or in other younger or older leaves of the same papaya plant. No particles were seen in the symptomless *N. benthamiana* plants.

Indirect ELISA was found to be sensitive enough for the detection of PMV in samples infected with the construct. The one plant of papaya and two *G. globosa* plants that showed positive absorbance values (Table 4-1. and 4-2.) were the same samples as those that tested positive in immunoblots and electron microscopy.

PMV infectious clone

PMV control

Figure 4-1. *Gomphrena globosa* inoculated with the PMV infectious clone showing local lesions.

Table 4-1. Ballistic inoculation of *G. globosa*, *N. benthamiana* and *C. papaya* with PMV infectious clone.

Host: *Gomphrena globosa*	1	2	3	4	5
Inoculum [a]	H_2O	uncut	*Not*I	*Kpn*I	PMV
Rate [b]	0/4	1/4	1/4	1/4	4/4
Symptoms [c]	0	LL- few	LL- few	LL- few	LL-many
Appearance [d]	0	9	9	8	7
Indexing- tissue blots, ELISA	0/4	1/4	1/4	1/4	4/4
Indexing- electron microscopy	0/4	0/4	1/4	1/4	4/4
Host: *Nicotiana benthamiana*	1	2	3	4	5
Inoculum [a]	H_2O	uncut	*Not*I	*Kpn*I	PMV
Rate [b]	0/4	0/4	0/4	0/4	4/4
Symptoms [c]	0	0	0	0	+Mo
Appearance [d]	0	0	0	0	11
Indexing- tissue blots, ELISA	0/4	0/4	0/4	0/4	4/4
Indexing- electron microscopy	0/4	0/4	0/4	0/4	4/4
Host: *Carica papaya*	1	2	3	4	5
Inoculum [a]	H_2O	uncut	*Not*I	*Kpn*I	PMV
Rate [b]	0/4	1/ 4	0/4	0/4	4/4
Symptoms [c]	0	Mo	0	0	Mo+
Appearance [d]	0	45	0	0	13
Indexing- tissue blots, ELISA	0/4	1/ 4	0/4	0/4	4/4
Indexing- electron microscopy	0/4	1/ 4	0/4	0/4	4/4

a= cDNA construct of PMV + 35S CaMV promoter in pGEM T Easy (conc=1µg/µl).
b= number of symptomatic plants/number of inoculated plants.
c= Symptom types Mo = mosaic, Mo+ = severe mosaic, LL= local lesion, 0= none.
d= time needed for appearance of symptoms following inoculation (in days).

Figure 4-2. Localized region with symptoms in papaya (arrow) ballistically inoculated with the infectious clone. Viral particles were observed in leaf dips made with material from that section of the leaf. No particles were found in leaf dips other areas of the same leaf.

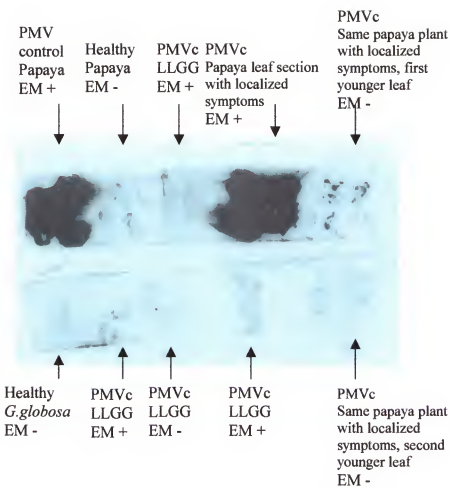

Figure 4-3. Tissue blots of plants ballistically inoculated with the PMV infectious clone. LLGG= local lesion on *G. globosa*, EM= electron microscopy, PMVc = PMV infectious clone.

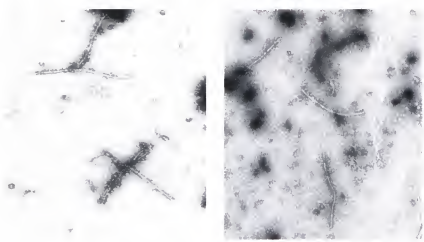

Figure 4-4. Viral particles stained with uranyl acetate observed in leaf dips of plants inoculated with PMV infectious clone. Magnification 30,000 X.

Table 4-2. Detection of PMV by I-ELISA[1] in extracts of plants ballistically inoculated with the infectious clone.

Sample[2] #	Inoculum concentration (μg/μl)	Plasmid	A_{405}	Symptoms	EM[3]
PMV control			0.678	Mosaic	+
Healthy control			0.002	None	-
G. globosa	1	*Not*I	0.256	Local lesions	+
G. globosa	1	*Kpn*I	0.347	Local lesions	+
C. papaya	1	uncut	0.638	Leaf area	+

1 I-ELISA=indirect ELISA.

2 Antigens used were extracts from leaf samples inoculated with the infectious clone of PMV, and with the PMV (ATCC) control. Antigens 1:10 in buffer and PMV antiserum 1:200 in buffer. The absorbance (A_{405}) value represents the mean of six wells.

3 EM= electron microscopy; + = filamentous particles observed; -+ no particles observed.

Mechanical Inoculation

Three of four plants of *Gomphrena globosa* with local lesions tested positive when mechanically inoculated with the infectious clone. Two papaya plants developed symptoms and became systemically infected after mechanical inoculation. Plants of *N. benthamiana* remained asymptomatic. Plants inoculated with the construct showed few symptoms, which developed slowly when compared to the controls. A few local lesions were observed on *G. globosa* when inoculated with the uncut plasmid, the plasmid cut with *Not*I, and the plasmid cut with *Kpn*I (Table 4-3., and 4-4.). All four controls inoculated with PMV were positive.

Local lesions in gomphrena plants inoculated with the different construct preparations all appeared after 15 days versus 5 days for the positive controls (Figure 4-5.). There was a marked difference in the size of the local lesions. Initially, the plants inoculated with the construct were smaller, compared to those inoculated with PMV. As time progressed these differences disappeared. In *G. globosa,* some of the plants showed initial signs of local lesion formation, but the lesions did not progress. Papaya plants inoculated with the PMV infectious clone showed systemic symptoms after 41 days (Figure 4-6.). The plants that showed symptoms were those inoculated with the plasmid that had either been uncut, cut with *Not*I (1/4), or cut with *Kpn*I (1/4) (Table 4-3. and 4-4.). All four controls were positive and showed symptoms after 20 days. Healthy controls were negative.

Table 4-3. Mechanical inoculation of *G. globosa*, *N. benthamiana* and *C. papaya* with PMV infectious clone.

Host: *G. globosa*	1	2	3	4	5
Inoculum[a]	H_2O	uncut	*Not*I	*Kpn*I	PMV
Rate [b]	0/4	1/ 4	1/ 4	1/ 4	4/ 4
Symptoms [c]	0	LL	LL	LL	LL
Appearance [d]	0	15	15	15	5
Indexing- tissue blots, ELISA	0/4	1/ 4	1/ 4	1/ 4	4/ 4
Indexing- electron microscopy	0/4	1/ 4	1/ 4	1/ 4	4/ 4
Host: *N. benthamiana*	1	2	3	4	5
Inoculum[a]	H_2O	uncut	*Not*I	*Kpn*I	PMV
Rate [b]	0/4	0/4	0/4	0/4	4/4
Symptoms [c]	0	0	0	0	Mo
Appearance [d]	0	0	0	0	10
Indexing- tissue blots, ELISA	0/4	0/4	0/4	0/4	4/4
Indexing- electron microscopy	0/4	0/4	0/4	0/4	4/4
Host: *C. papaya*	1	2	3	4	5
Inoculum[a]	H_2O	uncut	*Not*I	*Kpn*I	PMV
Rate [b]	0/4	1/ 4	1/ 4	1/ 3	4/4
Symptoms [c]	0	None	Mild Mo	Mild Mo	Mo
Appearance [d]	0	0	45	45	20
Indexing- tissue blots, ELISA	0/4	1/ 4	1/ 4	1/ 3	4/4
Indexing- electron microscopy	0/4	1/ 4	1/ 4	1/ 3	4/4

a= cDNA construct of PMV + 35S CaMV promoter in pGEM T Easy (conc=1µg/µl).
b= number of symptomatic plants/number of inoculated plants.
c= Symptom types Mo = mosaic, Mo+ = severe mosaic, LL= local lesion, 0= none.
d= time needed for appearance of symptoms following inoculation (in days).

Table 4-4. Detection of PMV by I-ELISA[1] in extracts of plants mechanically inoculated with the infectious clone.

Sample[2] #	Inoculum concentration (µg/µl)	Plasmid	A_{405}	Symptoms	EM[3]
PMV control			0.678	Mosaic	+
Healthy control			0.002	None	-
G. globosa	1	uncut	0.312	Local lesions	+
G. globosa	1	*Kpn*I	0.510	Local lesions	+
G. globosa	1	*Not*I	0.399	Local lesions	+
C. papaya	1	uncut	0.375	No symptoms	+
C. papaya	1	*Not*I	0.294	Mild mosaic	+
C. papaya	1	*Kpn*I	0.686	Mild mosaic	+

[1] I-ELISA=indirect ELISA

2 Antigens used were extracts from leaf samples inoculated with the infectious clone of PMV, and with the PMV (ATCC) control. Antigens 1:10 in buffer and PMV antiserum 1:200 in buffer. The absorbance (A_{405}) value represents the mean of six wells.

3 EM= electron microscopy; + = filamentous particles observed; -+ no particles observed.

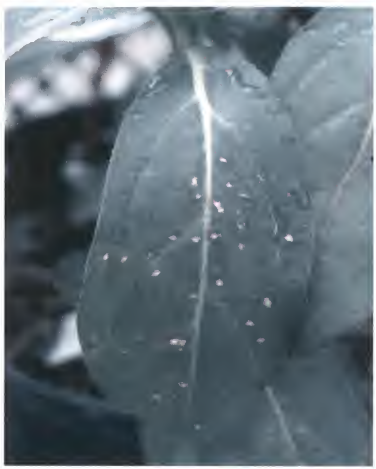

Figure 4-5. Local lesions on *G. globosa* mechanically inoculated with the PMV infectious clone.

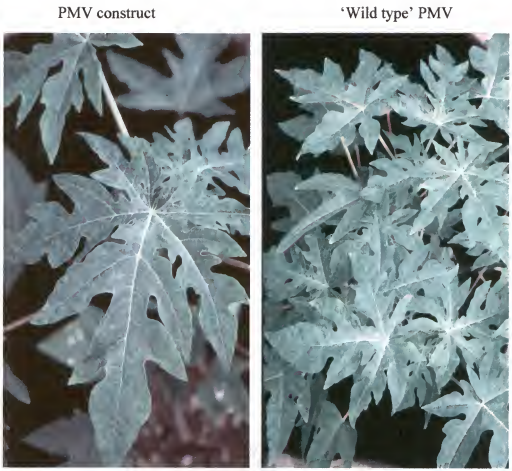

Figure 4-6. Comparison of symptoms caused by the PMV infectious clone versus those of 'wild type' PMV in papaya.

Indexing

The three mechanically inoculated plants of *G. globosa* with local lesions were positive when indexed with the chemiluminescent detection system. Three papaya plants indexed tested mildly positive in the tissue blot test when compared to the healthy control. Healthy controls were negative. PMV controls exhibited a strong positive reaction (Figure 4-7.). Particles were observed in one of three plants of *G. globosa* with local lesions. One plant of papaya without symptoms had particles typical of PMV, the papaya plants with symptoms all tested positive for particles when the sections of the leaves that had symptoms were checked. No virus particles were observed in *N. benthamiana* plants. When tested with ELISA, three plants of *G. globosa* tested positive for PMV: one inoculated with uncut plasmid, one inoculated with the plasmid cut with *Not*I and one inoculated with the plasmid cut *Kpn*I (Table 4-4.). Samples of papaya plants tested positive in ELISA: one inoculated with the uncut plasmid, one inoculated with material cut with *Not*I and one inoculated with the plasmid cut with *Kpn*I. Samples of *N. benthamiana* mechanically inoculated with the construct were all negative.

Ballistic Inoculation Light versus Dark

In this experiment no symptoms were observed in any of the plants inoculated using the ballistic method. There was some damage to the plant material. Young tissue,

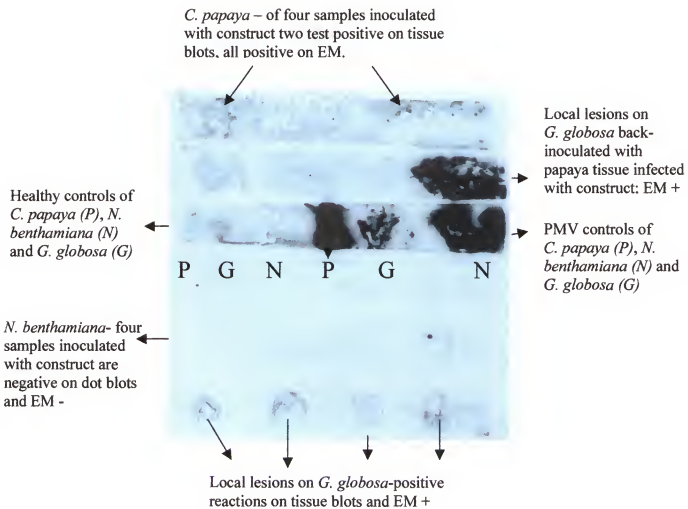

Figure 4-7. Tissue blots of mechanically inoculated plants of *C. papaya (P)*, *N. benthamiana* (N) and *G. globosa (G)*.

particularly of *N. benthamiana,* was the most damaged, even when placed far from the source of inoculum. PMV controls showed mosaic or local lesions accordingly, while healthy controls remained symptomless.

Mechanical Inoculations: Light Versus Dark

One plant of *G. globosa* and one plant of papaya developed symptoms of PMV after mechanical inoculation of plants kept in the dark for 24 hours prior to inoculation. Both the *G. globosa* plant showing local lesions and the papaya plant showing systemic symptoms were inoculated with the uncut plasmid. The plants were inoculated gently but tissue damage was evident, which might account for the low inoculation rate.

Back-Inoculations

Symptomatic material from plants previously inoculated with the construct (ballistic and mechanical) were used as the source of inoculum for subsequently inoculating G. *globosa, N. benthamiana* and *C. papaya* plants. The original host as well as the other two hosts were inoculated. Inoculated plants developed symptoms typical for PMV. Local lesions in back-inoculated gomphrena plants were evident after 4-5 days. PMV inoculated controls showed symptoms after 4 days. There were no differences in the reactions as compared to the controls. *N. benthamiana* plants became infected after being inoculated with inoculum from papaya or gomphrena plants.

Papaya plants inoculated with inoculum from local lesions of *G. globosa* or symptomatic leaves developed typical systemic symptoms of PMV after approximately 15 days, versus 13-14 days for the PMV inoculated controls.

Plants of *G. globosa* inoculated with papaya leaf inoculum developed symptoms after 4-5 days, and plants of *C. papaya* that were similarly inoculated developed symptoms after 9-10 days. Plants of *N. benthamiana* back- inoculated with the same inoculum developed symptoms in 11-12 days and tested positive for PMV. Tissue blots of inoculated plants were all positive (Figure 4-8.). By EM, viral particles were observed in all plants of *C. papaya*, *G. gomphrena*, and *N. benthamiana* that had been inoculated.

Mechanical Inoculations With Different Concentrations of PMV Clone

One papaya plant mechanically inoculated with 2 µg/µl of the infectious clone using the uncut plasmid as the source of inoculum developed symptoms after 32 days, and three papaya plants mechanically inoculated with 3 µg/µl of the infectious clone using the uncut plasmid as source of inoculum developed symptoms also after 32 days (Table 4-5.). Inoculum concentration appeared to have an effect on the frequency of infection. The PMV control developed symptoms after 21 days. Plants inoculated with 1 µg/µl did not develop symptoms. Symptoms included mild mosaic, vein clearing, and small chlorotic lesions. Some plants had symptoms in localized areas of the leaves, while others had the classical mosaic symptoms typical of PMV.

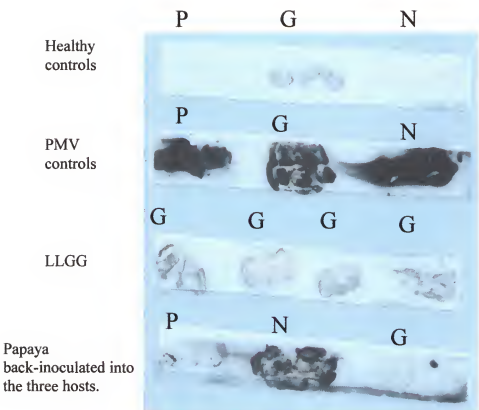

Figure 4-8. Tissue blots of plants back-inoculated with PMV infectious clone. LLGG=*G. globosa*, local lesions; *C. papaya*=P;*G. globosa*=G; *N. benthamiana*=N.

Table 4-5. The effect of concentration on the infectivity of the PMV infectious clone by restriction digest with *Kpn*I.

Host: *C. papaya*	1	2	3	4	5
Infectious clone [a] uncut	H_2O	1µg/µl	2µg/µl	3µg/µl	PMV
Frequency [b]	0/10	0/10	1/10	3/10	8/8
Symptoms [c]	0	0	Mo	Mo	Mo
Appearance [d]	0	0	32	32	21
Indexing- electron microscopy	0/10	0/10	1/10	3/10	8/8
Indexing- ELISA	10/10	10/10	10/10	3/10	8/8
Host: *C. papaya*	1	2	3	4	5
Infectious clone [a] cut with ***Kpn*I** at the 3' end	H_2O	1µg/µl	2µg/µl	3µg/µl	PMV
Frequency [b]	0	3/10	7/10	9/10	8/8
Symptoms [c]	0	Mo	Mo, Mo+	Mo, Mo-	Mo
Appearance [d]	0	32	26	24	21
Indexing- electron microscopy	0/10	3/10	7/10	9/10	8/8
Indexing- ELISA	0/10	3/10	7/10	9/10	8/8

a= cDNA construct PMV + 35S CaMV promoter in pGEM T Easy.
b= number of infected / number of inoculated plants.
c= Symptom types: Mo= mosaic, Mo+=severe mosaic, Mo-= mild mosaic, LL=local lesion.
d= time needed for symptom appearance following inoculation (in days).

When indexed with ELISA and EM, these tissues tested positive for the presence of PMV. Healthy controls remained without symptoms and tested negative for PMV (Table 4-6.). When papaya plants were mechanically inoculated with 1, 2 and 3 µg/µl of the infectious clone that had been cut with *Kpn*I at the 3' end, the inoculum concentration had a marked effect on the frequency of infection. Three out of ten plants inoculated with 1 µg/µl developed symptoms after 32 days. Seven out of ten plants inoculated with 2 µg/µl developed symptoms after 26 days and nine out of ten plants inoculated with 3 µg/µl developed symptoms after 24 days. The PMV controls developed symptoms after 21 days in all eight samples inoculated. Symptoms ranged from vein clearing (Figure 4-9.), mild chlorotic spotting, mild mosaic (Figure 4-10.) and a more pronounced mosaic symptom (Figure 4-11.). In several plants, symptoms were restricted to one area of the leaf while in other cases they were systemic. In general, mosaic symptoms were mild. Some plants appeared to be growing slower in relation to the others and the healthy controls; some had leaf constriction symptoms (Figure 4-12.). When indexed using ELISA and EM, the material tested positive for PMV (Table 4-5).

Particle concentration in leaf dips was lower when obtained from plants inoculated with the construct as compared to those inoculated with PMV. In ELISA tests, the reaction was stronger for the PMV control than in those of the construct. Negative controls remained without symptoms and tested negative for PMV.

Table 4-6. Detection of PMV by I-ELISA[1]. The plants were mechanically inoculated with the infectious clone to determine the effect of the concentration on the infectivity of the PMV infectious clone by restriction digest with *Kpn*I.

Sample[2] # (Tested 30 days after inoculation)	Inoculum concentration (μg/μl)	Plasmid	A_{405}	Symptoms	EM[3]
PMV control			2.600	mosaic	+
Healthy control			0.005	none	-
69	2	uncut	0.098	chlorotic spotting	+
76	3	uncut	0.130	mild mosaic	+
78	3	uncut	0.111	chlorotic spotting	+
80	3	uncut	0.145	vein clearing	+
83	1	cut	0.083	chlorotic spotting	+
88	1	cut	0.088	chlorotic spotting	+
91	1	cut	0.073	mild mosaic	+
93	2	cut	0.074	chlorotic spotting	+
94	2	cut	0.083	mild mosaic	+
98	2	cut	0.124	mild mosaic	+
99	2	cut	0.100	mild mosaic	+
100	2	cut	0.084	leaf constrictions	+
101	2	cut	0.075	severe mosaic	+
102	2	cut	0.075	leaf constrictions	+
103	3	cut	0.243	very mild mosaic	+
104	3	cut	0.263	mild mosaic	+
105	3	cut	0.411	very mild mosaic	+
106	3	cut	1.42	mild mosaic	+
107	3	cut	0.070	no symptoms	-
108	3	cut	0.734	mild mosaic	+
109	3	cut	0.660	mild mosaic	+
110	3	cut	0.665	mild mosaic	+
111	3	cut	0.085	mild mosaic	+
112	.3	cut	0.081	mild mosaic	+

[1] I-ELISA=indirect ELISA

2 Antigens used were extracts from leaf samples inoculated with the infectious clone of PMV, and with the PMV (ATCC) control. Antigens 1:10 in buffer and PMV antiserum 1:200 in buffer. The absorbance (A_{405}) value represents the mean of six wells.

3 EM= electron microscopy; + = filamentous particles observed; -+ no particles observed.

Figure 4-9. Papaya leaf inoculated with 3 μg/μl of uncut infectious clone of PMV showing vein clearing.

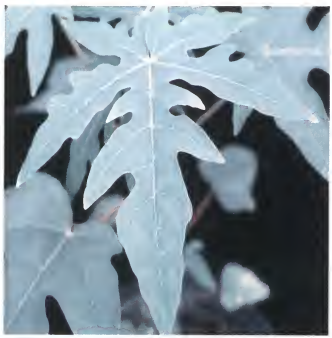

Figure 4-10. Papaya plant inoculated with 3 μg/μl of PMV clone cut with *Kpn*I showing mild mosaic systemic symptoms.

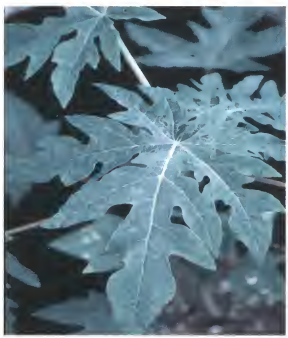

Figure 4-11. Systemic symptoms on older papaya plants inoculated with 2 µg/µl of PMV infectious clone cut with *Kpn*I.

Figure 4-12. Papaya inoculated with infectious clone showing leaf constriction symptoms.

The highest infection rates in plants inoculated with the uncut infectious clone occurred when inoculated at an inoculum concentration of 3 μg/μl, followed by 2 μg/μl. The highest infection rates in plants inoculated with the *Kpn*I cut infectious clone occurred at an inoculum concentration of 3 μg/μl, followed by 2 μg/μl, and 1 μg/μl (Table 4-5.).

Discussion

The CaMV promoter-driven PMV cDNA was shown to be infectious in *G. globosa*, *C. papaya*, but not in *N. benthamiana*. The ballistic inoculation method was very inefficient. The low infection frequency may have been due to the concentration of the DNA used in the inoculum. The extensive tissue damage that occurred with this method might have hindered further replication and cell-to-cell movement of the construct. Mechanical inoculations were more successful possibly because the plants only suffered limited damage during inoculation. Inoculum concentration had a marked effect on the frequency of infection. Higher concentrations of inoculum resulted in a higher incidence of infection and a faster rate of symptom development.

Disease progress resulting from the infectious cDNA was slower in relation to movement within the plant when compared to the PMV control. An abundance of viral particles were observed by EM in the PMV control tissues, but less in the material inoculated with the infectious clone. The fact that symptoms took longer to appear on the plants inoculated with the infectious clone may indicate that although the clone is infectious, it might be slightly defective in terms of movement and systemic spread. This

might explain the differences seen in the symptom expression of plants inoculated with the construct when compared to PMV. Increasing the inoculum levels of the infectious clone resulted in increased virus spread and reduced the amount of days to obtain symptoms.

The back-inoculated plants did not differ in symptom expression or time of onset of symptoms from the plants inoculated with PMV. The systemic symptoms and local lesions developed at the same rate at which they occur for PMV. One can speculate that perhaps there were differences in the sequences of the infectious clone constructed when compared to the 'wild type' PMV. Once the virus was *in planta* it may have repaired whatever differences might account for the response observed and that allowed it to function as effectively as the native virus. Another possibility stems from the fact that the construct was developed from only one copy of the virus. which could have made it less effective as a pathogen since in plants, viruses exist as a population of variants.

Other limiting factors to the movement of the infectious clone from cell to cell, as seen in some of the plants that show symptoms only on sections of leaves, might be changes in the sequence that might have affected the triple gene block, which in potexviruses is associated with cell to cell movement.

AbouHaidar and Bancroft (1978b) have indicated that the initiation of assembly for PMV happens within 200 bases of the 5' end and that there is probably an interaction between a 14S species of the CP and the 5' end of the viral RNA. In these experiments, any nucleotide changes that may have occurred during the PCR process when the cDNA was constructed may have partially affected the initiation of assembly and may retard the elongation, a step also dependent on RNA and coat protein interactions.

Dagless et al., (1997) have found that TMV-U1 cDNAs driven by the 35S CaMV promoter were able to infect some plants but not others when they did mechanical inoculations. Weber et al. (1992) found that although TMV-L driven by the 35S promoter was infectious at a concentration of 1 μg/μl when inoculated in *Chenopodium quinoa*, higher concentratons (up to 100 μg/μl) failed to infect other hosts. Low infectivity has been also observed after manual inoculation of plants using the intact plasmid when using cucumber mosaic virus (Ding et al., 1995) and tomato mosaic virus (Weber et al., 1992).

In this study, similar results were observed. Lower infectivity was observed when using lower concentrations of the 35S cDNA promoter driven PMV. No symptoms were observed in one natural host, *N. benthamiana*. Our results show that after inoculation the infections developed slowly. This has been also reported by Dagless et al., (1992) with construct 35S-TMV-U1.

The variability obtained when inoculating plants with the PMV infectious clone via ballistic or mechanical inoculation shows similarities to the ones observed by others working with cDNAs of viruses driven by the 35S promoter. With the information obtained from this study, improvements can be made in order to achieve better results when using a 35S driven PMV construct.

CHAPTER 5
SUMMARY AND CONCLUSIONS

Two separate approaches were attempted in order to develop full-length cDNA clones of PMV. The first approach involved the assembly of two subclones of PMV from two segments of PMV that were obtained from PCR. This approach did not work. The second approach involved synthesis of full-length cDNA using Superscript^{TM}II. This approach yielded full-length cDNA.

Attempts were made to clone the full-length cDNA. Two of the 6.7 kbp clones were obtained and sequenced in order to determine sequence homology to the known sequence for PMV. The second clone sequenced (clone 10) contained the complete sequence and had a high degree of homology with PMV. This clone was selected for subsequent experiments to produce an infectious clone.

Using PCR, the 35S promoter was amplified from the plasmid vector pCambia 1302. A full-length copy of PMV was amplified from clone #10 and 20 T's were added to the 3' end. An overlapping PCR reaction was used to add the CaMV 35S promoter to the 5' end of the PMV cDNA. The final construct measured 7.2 kbp and contained an *Xba*I site at the beginning followed by the 35S CaMV promoter directly attached to the 5' end of PMV and ending with a poly A_{20} tract with a *Kpn*I site at the 3' end.

The results of experiments with ballistic inoculation indicated that the inoculation methods used were very inefficient. The low infection rate may have been be due to the

concentration of the DNA used in the inoculum. The tissue damage that occurred by using this method may have impeded further replication and cell-to-cell movement of the construct.

Mechanical inoculations were more successful possibly because the plants only suffered limited damage. The PMV construct was infectious, but the disease progress was slower with respect to movement within the plant when compared to the PMV control.

Electron microscopy of affected tissues indicated an abundance of particles in the PMV inoculated control tissues and comparatively few in the material inoculated with the infectious clone. Some of the inoculated plants developed the typical PMV mosaic symptoms. In grids of leaf dips made with PMV inoculated plants there were approximately 3-4 particles per each window on that grid. In grids of leaf dips of plants inoculated with the infectious PMV clone, there was approximately one particle every two windows of the grid.

The back-inoculated plants did not differ in symptom expression or time of onset of symptoms from the positive control plants. The systemic symptoms and local lesions developed at the same rate in the back-inoculated plants as they did in the PMV controls inoculated. The symptom expression in the three hosts may indicate that once the virus is *in planta* it might repair whatever damage it has initially which allows it to function as effectively as its PMV counterpart. The construct prepared is only one copy of the virus. In nature viruses exist as a population is which might include variants.

Results of the experiments comparing the effectiveness of keeping the plants in the dark for 24 hours prior to inoculation indicate that this might offer an advantage over

the plants that were kept in the light as reported by Yarwood and Fulton, (1967).

Inoculum concentration had a marked effect on the rate of infection. Higher concentrations of inoculum yielded more infected plants and these developed symptoms faster as compared to lower concentrations of inoculum. The plants that were positive for the PMV construct developed milder symptoms than the PMV control. Symptoms ranged from mild mosaic to mild shoestring or only parts of leaves affected.

Symptoms took longer to appear on the plants inoculated with the infectious clone. This might indicate that although the clone is infectious, it might be slightly defective in terms of cell-to-cell movement and systemic movement within the plant. This might explain the differences seen in the symptom expression of plants inoculated with the construct when compared to those due to PMV.

The results obtained in this study might suggest that, after the infectious clone replicates for a while in a plant, it evolves to a more efficient or virulent pathogen. It may be possible that through mutation and selection during replication *in planta* the cloned virus reverts to its more efficient 'wild type' form.

The results indicate that the efficiency of the infectious clone is limited. Several steps can be taken to improve this in further studies. First, once a cDNA is obtained and clones are produced, these should be completely sequenced and compared for sequence homology with the PMV sequences available in databases at the moment. Clones that have the greatest homology should be used to continue experiments. Preferably, a mixture of clones should be developed to increase the possibility of efficiency of the infectious clone. Second, once the 35S CaMV promoter is added, using the method described, the sections containing the newly attached regions should be sequenced to

determine if the regions are identical to the ones originally attached. Work should continue only with those that have identical sequences. Third, in order to ascertain at the DNA level that the virus recovered in systemic primary infections or back-inoculations is the original clone, silent mutations should be introduced in the clone. These are some of the steps that can be taken in the future in order to improve the previous research.

In summary, the results obtained when inoculating host plants with the infectious construct via ballistic or mechanical inoculation indicated that a number of possibilities for this system. In the future, improvements to the existing construct can provide a simple and reliable method for the mechanical inoculation of PMV cDNAs driven by the 35S promoter. Obtaining an infectious clone of papaya mosaic virus opens the possibility to develop it as a vector for the expression of foreign genes in papaya. The use of PMV as a vector may have several advantages over the use of transgenic plants, among them: the expression is limited only by the host range of the virus vector and not by the cultivar as is the case with transgenic plants; the production of pathogen-derived resistance may be readily adapted to new papaya varieties; and the expressed genes might be modified quickly by recombinant DNA methods to counter changes in the PRV in the field. In addition, the effect can be tested without going through the process of plant transformation. The development of PMV as a vector for the expression of PRV genes and/or sequences in papaya for pathogen-mediated resistance in Florida and the Caribbean could greatly increase papaya production. These alternatives for control would give papaya growers a solution to a limiting production problem.

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BIOGRAPHICAL SKETCH

Margarita Ferwerda-Licha was born in Rio Piedras, Puerto Rico, on December 23, 1956. She completed her undergraduate degree from the University of Puerto Rico, Mayaguez Campus, in 1976 with a B.S. in Horticulture. She enrolled in the Department of Plant Pathology where she obtained the Master of Science degree under the direction of Dr. Julio Bird in 1979. She has worked for the Department of Plant Pathology and the Department of Chemistry at the University of Puerto Rico at Mayaguez and also for the USDA-ARS-Northern Plains Area. She began her Ph.D. research in 1996 in the Department of Plant Pathology at the University of Florida under the direction of Dr. Ernest Hiebert and Dr. Michael J. Davis. She expects to complete this program in August of 2000 and receive her Ph.D. degree. Margarita is a member of the American Phytopathological Society, Gamma Sigma Delta, and Alpha Zeta. Upon completion of her Ph.D. she will be joining the Crop Protection Department as a Plant Virologist at the University of Puerto Rico in Mayaguez, Puerto Rico.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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